ON CROSS REACTIONS OF IMMUNE SERA TO AZOPROTEINS

II. Antigens with Azocomponents Containing Two Determinant Groups

By K. LANDSTEINER, M.D., and J. van der SCHEER

(From the Laboratories of The Rockefeller Institute for Medical Research)

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The question of serological cross reactions has recently been commented upon by other authors and ourselves (1–5). To recapitulate briefly, an immune serum may exhibit cross reactions by virtue of an antibody able to combine with substances more or less closely related to the homologous antigen in chemical structure, or it may contain multiple antibodies, differing in specificity, some of which cross react with certain heterologous antigens. The appearance of several antibodies after immunization with a particular antigenic material may depend upon the presence in the latter of different antigenic molecules, or upon the existence, in a single molecule, of more than one determinant group; moreover, as has been shown in our studies on azoproteins (4), multiple antibodies varying somewhat in specificity may be produced in response to one determinant structure in cases where the antigen does not contain divers chemical groupings that in part are shared by the reacting heterologous antigens. To illustrate the latter, an immune serum produced by injecting an azoprotein made from *m*-aminobenzenesulfonic acid was found to contain several antibody fractions reacting differently towards antigens made from *o*-aminobenzenesulfonic, *m*-aminobenzoic and *m*-aminophenylarsenic acids. Similar results were found in other instances and may well be expected to apply to natural antigens and their antibodies.

One can assume therefore that, by and large, in using any immune serum one deals not with a single antibody but with a mixture of somewhat different immune bodies (*cf.* Heidelberger and Kendall (6), Goodner and Horsfall (7 *a*), Lee *et al.* (7 *b*), Hooker (2)). In the case of

multiple antibodies produced by single natural antigens, the familiar idea is that these antibodies are formed in response to and are indicative of different determinant groups in the antigenic molecule.

Evidence for this concept is offered by the observation that immune sera engendered by azoproteins contain different antibodies severally acting on the original protein, on the hapten and, probably, on the conjugate[1] (1, 8, 4).

Another example, somewhat better defined with regard to the underlying determinant structure in the antigen, is the following in which the antigen used was ovalbumin to which had been attached two sorts of determinant groups. Crystalline egg albumin was treated with picryl chloride (9) in order to introduce picryl radicals, and the product of the reaction was coupled in the usual manner with a diazonium compound, namely diazotized *p*-aminophenylarsenic acid. Following injection of this material into rabbits two sorts of antibodies were demonstrable by absorption experiments, one reacting with picryl proteins, the other with azoproteins made from *p*-aminophenylarsenic acid. Similar results would probably be obtained if two or more azocomponents were to be attached to protein. Antibodies combining with both the phenylarsenic acid and the trinitrophenyl (picryl) residues could not be definitely ascertained.

Since in these instances the structure of the antigens with regard to the relation of the determinant groups to each other is not fully established, there still remained for investigation the antibody response to simple haptens of known constitution possessing different groups each of which has been shown to serve as a serological determinant. With this in view, aromatic amino compounds containing two different acid groupings were synthesized and used for preparing conjugated antigens. The immune sera obtained by injection of these azoantigens were examined for the presence of multiple antibodies by means of "partial" antigens made from compounds containing only one of the acid groups.

Materials and Methods

3-Amino, 5-Carboxysuccinanilic Acid.—3,5-Dinitrobenzoic acid was reduced in alcoholic solution with ammonium sulfide by the method of Flürscheim (10) to 3-amino, 5-nitrobenzoic acid. For purification an aqueous solution of the ammonium salt was strongly acidified by adding concentrated HCl and after filtration the free amino compound was precipitated by addition of the required amount of NaOH. M.p. after recrystallization from water 208°C. (11).

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[1] This might be taken to indicate the existence, in such sera, of different binding groups in a single antibody.

3-Amino, 5-nitrobenzoic acid and succinic acid were fused together for 15 minutes at 165° (12). The precipitate obtained by acidification of an alkaline aqueous extract of the product was recrystallized from water. Yield 70 per cent of theory. Needles, m.p. 212–213°C. Titration: 0.280 gm. neutralized 9.9 cc. N/10 NaOH. Calculated for 3-nitro, 5-carboxysuccinanilic acid ($C_{11}H_{10}O_7N_2$) 9.92 cc.

This compound was reduced with ferrous sulfate as described previously (12). After filtration the solution was made weakly acid to Congo red and the precipitate was recrystallized from water. Yield 75 per cent. Needles, m.p. 139–140°C. After drying at 50° *in vacuo* over $CaCl_2$: calculated for 3-amino, 5-carboxysuccinanilic acid ($C_{11}H_{12}O_5N_2$): N 11.11; found 11.06.

m-Aminosuccinanilic Acid.—*m*-Nitrosuccinanilic acid, prepared by fusion of *m*-nitraniline and succinic acid, was reduced with ferrous sulfate to the amino compound as described for the para compound (12). Yield 70 per cent of theory. Needles, m.p. 152°C. after recrystallization from water. Calculated for $C_{10}H_{12}O_3N_2$: N 13.45; found 13.37.

(3-Amino, 5-Succinylaminobenzoyl)-p-Aminobenzoic Acid.—Following the methods described below for the preparation of the corresponding As compounds (3,5-dinitrobenzoyl)-*p*-aminobenzoic acid was obtained by treating *p*-aminobenzoic acid with 3,5-dinitrobenzoyl chloride. After recrystallization from 50 per cent acetone (needles, no m.p. up to 250°C.), it was reduced by means of ammonium sulfide to (3-nitro, 5-aminobenzoyl)-*p*-aminobenzoic acid, which was recrystallized by acidifying a hot solution of the sodium salt in 50 per cent alcohol with a slight excess of N HCl and cooling to room temperature. Orange colored platelets, no m.p. up to 250°C. Calculated for $C_{14}H_{11}O_5N_3$: N 13.90; found 13.71.

A finely ground mixture of 3 gm. of this substance and 6 gm. of succinic anhydride, was kept at 130–135°C. for 15 minutes. The reaction mixture was dissolved in hot water by addition of sodium hydroxide and the substance precipitated by acidification with hydrochloric acid. It was recrystallized from a large volume of 50 per cent alcohol. Yield 2.6 gm. Clusters of needles, no m.p. up to 250°C. Calculated for (3-nitro, 5-succinylaminobenzoyl)-*p*-aminobenzoic acid ($C_{18}H_{15}O_8N_3$): N 10.47; found 10.51.

This nitro compound was reduced with ferrous sulfate and precipitated from the filtered solution by making weakly acid to Congo red. Recrystallized from a large volume of 50 per cent alcohol. Yield 75 per cent. Needles, decomposes at about 250°C. Calculated for $C_{18}H_{17}O_6N_3$: N 11.32; found 11.23.

(3-Amino, 5-Succinylaminobenzoyl)-p-Aminophenylarsenic Acid.—To 11 gm. of arsanilic acid dissolved in 300 cc. of 10 per cent sodium bicarbonate solution 17 gm. of 3,5-dinitrobenzoyl chloride dissolved in 200 cc. of chloroform were added in 5 portions with vigorous shaking during 1 hour. The precipitate formed upon acidification with HCl was dried and extracted several times with ether. Yield 19 gm. It was purified further by dissolving in 1 liter of boiling water and 20 cc. of concentrated ammonium hydroxide, and precipitating with acetic acid. Needles. Calculated for (3,5-dinitrobenzoyl)-*p*-aminophenylarsenic acid ($C_{13}H_{10}O_8N_3As$): N 10.22; found 10.12.

The substance was dissolved in hot water by addition of a slight excess of ammonia, and there was added 90 per cent of the quantity of ammonium sulfide (see Flürscheim (10)) required for the reduction of one nitro group. After being heated for 1 hour the solution was evaporated to dryness *in vacuo* and the residue was extracted with boiling water. To the extract was added one-fifth volume of concentrated HCl, and after cooling in a freezing mixture for ½ hour it was filtered and adjusted to weak acidity to Congo red by addition of concentrated NaOH. The precipitate was recrystallized from a large volume of 30 per cent alcohol. Yield 6 gm. from 10 gm. of the dinitro compound. Clusters of needles. Calculated for (3-nitro, 5-aminobenzoyl)-*p*-aminophenylarsenic acid ($C_{13}H_{12}O_6N_3As$): N 11.02; found 10.94.

10 gm. of the nitroamino compound were finely ground and refluxed for 3 hours with 150 cc. of absolute alcohol and 10 gm. of succinic anhydride. After cooling the material was filtered off and freed from unchanged nitroamino compound by dissolving in 200 cc. water by means of NaOH and subsequently adding 60 cc. of concentrated HCl, the amino compound staying in solution. The precipitate was purified after it had been dissolved in a small amount of water with NaOH by precipitation with HCl and crystallization from a large volume of 50 per cent alcohol. Yield 6 gm. Oval shaped platelets. Calculated for (3-nitro, 5-succinylaminobenzoyl)-*p*-aminophenylarsenic acid ($C_{17}H_{16}O_9N_3As$): N 8.73, As 15.57; found N 8.74, As 15.34.

4 gm. of the above substance were finely ground and suspended in a mixture of 100 cc. of 95 per cent ethyl alcohol and 100 cc. of N HCl, and 8 gm. of zinc dust were slowly added with continuous stirring over a period of about 1 hour with further addition of 20 cc. of N HCl when the solution became weakly acid. After the nitro compound had disappeared the solution was filtered and 8 gm. of sodium acetate were added. The precipitate was washed twice with water and redissolved in 100 cc. of water by addition of N NaOH. The solution was made strongly acid to Congo red by addition of concentrated HCl and clarified by centrifugation. After neutralization of the solution and addition of sufficient ammonia to redissolve the precipitate formed, ammonium sulfide was added until a flocculent precipitate of zinc sulfide was formed. After removal of zinc sulfide acidification to weak Congo red reaction gave an amorphous precipitate which crystallized upon heating on the steam bath (rosettes of oval platelets). Yield 2.4 gm. For further purification 1 gm. of substance was dissolved in 50 cc. of water by addition of N NaOH; the neutral solution was decolorized by heating with charcoal. The hot filtrate was acidified with 5 cc. glacial acetic acid; crystallization took place slowly upon cooling. Calculated for (3-amino, 5-succinylaminobenzoyl)-*p*-aminophenylarsenic acid ($C_{17}H_{18}O_7N_3As$): N 9.31, As 16.60; found N 9.15, As 16.48.

sym. Aminoisophthalyl Glycine-d,l-Leucine.—A solution of 5 gm. of sym. nitroisophthalic acid in 100 cc. of dry ether was cooled to 0° and shaken with 5 gm. of finely ground PCl_5 for about 45 minutes at low temperature until all the PCl_5 was used up. After evaporation to dryness at 60° under reduced pressure the

residue was extracted twice with 25 cc. of boiling benzene. Addition of 3 volumes of dry petrol ether to the benzene solution gave a crystalline precipitate of the monochloride which was recrystallized from 20 parts of dry toluene. Yield 1.2 gm. Platelets, m.p. 123–124°, with evolution of gas. Calculated for sym. nitroisophthalic acid monochloride ($C_8H_4O_5NCl$): Cl 15.44; found 15.29.

To a dry chloroform solution (50 cc.) of glycine ethyl ester prepared from 5 gm. of hydrochloride were added 5 gm. of dry dimethylaniline and 5 gm. of sym. nitroisophthalic acid monochloride dissolved in 125 cc. of dry chloroform. The mixture was allowed to stand overnight at room temperature and after removing a precipitate of glycine ester hydrochloride the solution was shaken with 200 cc. of N/2 HCl. The precipitate was filtered off, washed with water and recrystallized from 50 per cent alcohol. Yield 5 gm. Clusters of needles, m.p. 203–204°C. Calculated for sym. nitroisophthalyl monoglycine ethyl ester ($C_{12}H_{12}O_7N_2$): N 9.46; found 9.37.

2.95 gm. of sym. nitroisophthalyl monoglycine ethyl ester in a mixture of 60 cc. of dry benzene and 20 cc. of dry ether were shaken with 3 gm. of PCl_5 for ½ hour at room temperature and finally a short time at 37°C. When practically all the PCl_5 had disappeared the solution was filtered and the substance was precipitated by adding 3 volumes of dry petroleum ether. Yield 2.4 gm. Platelets, m.p. 127–128°C. Calculated for chloride of sym. nitroisophthalyl monoglycine ethyl ester ($C_{12}H_{11}O_6N_2Cl$): Cl 11.29; found 11.36.

A solution of 3 gm. of this substance in 50 cc. of dry ethyl acetate was mixed with a solution of *d,l*-leucine methyl ester, prepared from 3.5 gm. of hydrochloride, in 75 cc. of dry ethyl acetate. After standing at room temperature overnight the solution was shaken successively with dilute HCl, dilute aqueous $NaHCO_3$ and water. After drying with Na_2SO_4 the ethyl acetate was removed by evaporation and the syrup was dissolved in 40 cc. of methyl alcohol. 20 cc. of N NaOH were added and the saponification was allowed to proceed for 1 hour at room temperature. After neutralization the solution was evaporated to dryness, the residue was dissolved in a small volume of water and made acid to Congo red by addition of 5 N HCl. After several days in ice box and occasional rubbing, the sticky precipitate crystallized in hair-like needles. The substance was redissolved in water with alkali and reprecipitated with acid. It was filtered off in the ice box and was dried *in vacuo* over P_2O_5. Yield 3 gm. M.p. 125–126°C. Calculated for sym. nitroisophthalyl glycine-*d,l*-leucine ($C_{16}H_{19}O_8N_3$): C 50.39, H 5.02, N 11.02; found C 50.48, H 5.08, N 10.90.

3.8 gm. of the nitro compound dissolved in 10 cc. of water by addition of ammonia were added to a hot solution of 18.7 gm. of ferrous sulfate (7 aq.) in 50 cc. of water. 16 cc. of 28 per cent ammonia solution were added in 5 portions over a period of 10 minutes. After ½ hour's heating on the steam bath, the solution was filtered and evaporated *in vacuo* to about 25 cc.; a large volume of alcohol was added. Ammonium sulfate was removed by filtration and the solution was evaporated *in vacuo*. After redissolving the residue in absolute methyl alcohol and evaporating to dryness, the substance was taken up in 30 cc. of absolute

methyl alcohol. The solution was filtered and the substance precipitated with 90 cc. of dry ether. Upon dissolving the precipitate in a large amount of boiling absolute ethyl alcohol and adding sufficient ether to the cooled solution to give a slight turbidity, crystallization took place in the ice box. The free amino compound obtained by acidification of a concentrated aqueous solution of the ammonium salt was a syrup which did not crystallize. It was washed several times with cold water and solidified on drying *in vacuo* over P_2O_5. Calculated for sym. aminoisophthalyl glycine-*d*,*l*-leucine ($C_{16}H_{21}O_6N_3$): C 54.66, H 6.07, N 11.95; found C 54.50, H 6.19, N 11.67. Titration: 0.0667 gm. in 50 per cent alcohol neutralized 18.85 cc. N/50 $Ba(OH)_2$. Calculated 19.00 cc.

sym. Aminoisophthalyl Glycine-d,l-Phenylalanine.—The chloride of sym. nitroisophthalyl glycine ethyl ester was condensed with the ethyl ester of *d*,*l*-phenylalanine in dry ethyl acetate solution as described above. After washing the solution with dilute HCl, etc., and drying over Na_2SO_4, it was evaporated to dryness *in vacuo* and the residue was recrystallized from methyl alcohol. Yield 3.8 gm. from 3 gm. of the chloride. Hair-like needles, m.p. 138°C.

To a solution of 3.7 gm. of this ester in 70 cc. of acetone were added 16 cc. of N NaOH, and after 1 hour the solution was neutralized and evaporated to dryness. The residue was dissolved in a small amount of water and after acidification to Congo red the oil which separated was stirred with chloroform until crystallization took place. After acidification of a very dilute alkaline aqueous solution and removal of a small amount of tarry material the substance crystallized slowly at room temperature. Yield 2.5 gm. Clusters of curved needles, m.p. 211–212°C. Calculated for sym. nitroisophthalyl glycine-*d*,*l*-phenylalanine ($C_{19}H_{17}O_8N_3$): N 10.11; found 10.01.

The reduction of the nitro compound was carried out with ferrous sulfate as described above. After removing most of the ammonium sulfate with alcohol, the solution was evaporated to dryness and the residue dissolved in a small amount of water. Upon acidification an oil separated which slowly crystallized in microscopic platelets. The substance was recrystallized from a large volume of water. After drying at 90° *in vacuo* it softened at 139°, and decomposed at 178°. Calculated for sym. aminoisophthalyl glycine-*d*,*l*-phenylalanine ($C_{19}H_{19}O_6N_3$): N 10.90; found 11.03.

m-Nitrobenzoyl and m-Aminobenzoyl Derivatives of Glycine and d,l-Leucine.—These were prepared by shaking a solution of the amino acids in 10 per cent $NaHCO_3$ (the leucine was first dissolved in water by addition of a slight excess of N NaOH) with a chloroform solution of *m*-nitrobenzoyl chloride (1.25 mols to each mol of amino acid) and reduction of the nitro compounds with ferrous sulfate as described previously.

m-Nitrobenzoyl glycine: Recrystallized from 50 per cent alcohol. Needles, m.p. 165–166°C. Titration: 200 mg. dissolved in 50 per cent alcohol required for neutralization 8.9 cc. N/10 NaOH. Formula $C_9H_8O_5N_2$ requires 8.92 cc.

m-Aminobenzoyl glycine: Recrystallized from water. Platelets, m.p. 191–192°C. Calculated for $C_9H_{10}O_3N_2$: N 14.43; found 14.25.

m-Nitrobenzoyl *d,l*-leucine: Recrystallized from benzene. Long needles, m.p. 132–133°. Titration: 200 mg. dissolved in 50 per cent alcohol required for neutralization 7 cc. N/10 NaOH. Formula $C_{13}H_{16}O_5N_2$ requires 7.14 cc.

m-Aminobenzoyl *d,l*-leucine: Recrystallized from water. Platelets, m.p. 157–158°C. Calculated for $C_{13}H_{18}O_3N_2$: N 11.19; found 11.12.

Immunization.—Rabbits were injected intravenously with 2 cc. of a suspension containing 2.5 mg. of azostromata (from horse blood) (4) in 1 cc. After two to four courses of 6 daily injections given at intervals of 1 week, the animals were bled 7 days following the final injection.

Tests.—Antigens used for the tests were made by coupling the diazonium compounds with chicken serum as described (13). 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), tr. (strong trace), ±, ±, +, +±, ++, etc.

Absorption Experiments.—A suspension of azostromata was centrifuged and the sediment was mixed with concentrated or diluted immune serum. The suspension was stirred occasionally during 2 hours at room temperature, after which the stromata were removed by centrifuging (4).

EXPERIMENTAL[2]

The sera made with two of the four antigens, those from aminocarboxysuccinanilic acid (SC) and (aminosuccinylaminobenzoyl)-*p*-aminobenzoic acid (SB), contained, curiously, antibodies that were entirely or almost completely directed towards only one of the acid groups, namely the succinanilic acid residue, and therefore did not yield the desired information on the alternative whether there would be formed antibodies corresponding to the structure of the molecule as a whole or more than one antibody, each adjusted to one of the determinant groups. Thus the SC antisera precipitated with nearly equal intensity the SC azoprotein and an azoprotein made from *m*-aminosuccinanilic acid (S) but out of four SC sera only one, not the most potent, gave a weak reaction with an azoprotein prepared from *m*-amino-

[2] For brevity the substances used for preparing the azoantigens, as well as these antigens and the corresponding antisera, will be designated throughout as follows: 3-amino, 5-carboxysuccinanilic acid (SC), *m*-aminosuccinanilic acid (S), (3-amino, 5-succinylaminobenzoyl)-*p*-aminobenzoic acid (SB), (3-amino, 5-succinylaminobenzoyl)-*p*-aminophenylarsenic acid (SA), *p*-aminophenylarsenic acid (A), sym. aminoisophthalyl glycine-*d,l*-leucine (GIL), sym. aminoisophthalyl glycine-*d,l*-phenylalanine (GIPh), *m*-aminobenzoyl glycine (G), *m*-aminobenzoyl *d,l*-leucine (L).

benzoic acid. The relation in chemical structure of the compounds is seen from the formulae given below.

Although directed towards the succinanilic acid group of the SC antigen, the SC immune sera were found by inhibition tests not to be identical with sera made with S antigen. In such tests the reactions of SC sera on SC or S antigens were markedly more inhibited by 3-nitro, 5-carboxysuccinanilic acid than by *m*-nitrosuccinanilic acid, while this was not the case with S immune sera. The homologous reaction of SC sera was not significantly inhibited by *m*-nitrobenzoic acid.

TABLE I

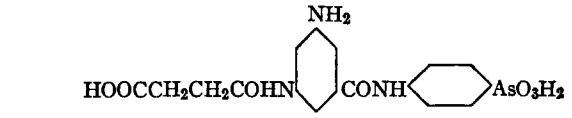
0.1 cc. of the immune sera was added to 0.2 cc. of the antigen solutions. Readings after 1 hour at room temperature.

Immune sera	Test antigens														
	SA			S			A			SC			SB		
	1:100	1:500	1:2500	1:100	1:500	1:2500	1:100	1:500	1:2500	1:100	1:500	1:2500	1:100	1:500	1:2500
SA	+±	+++	+	+++	++++	+	tr.	++	+	++±	+++	+	++	+++	+
S	f.tr.	±	f.tr.	+±	+++	±	0	0	0	+	+±	±	tr.	±	f.tr.
A	0	±	tr.	0	0	0	±	++±	+	0	0	0	0	0	0
SC	f.tr.	+	±	+	+++	+	0	0	0	+	++±	+±	tr.	+	tr.

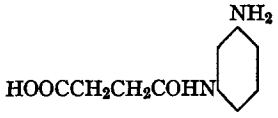
Essentially similar in all respects were the results observed with (3-amino, 5-succinylaminobenzoyl)-*p*-aminobenzoic acid antigen. The sera failed to precipitate *p*-aminobenzoic acid antigen but reacted with S antigen, even more intensely than with SB antigen, while immune sera made with *p*-aminobenzoic acid antigen gave distinct precipitation with SB antigen. Inhibition tests as described above again demonstrated the greater affinity of the homologous substance and clearly showed a difference between SB and S immune sera.

In distinction to the experiments described, results answering the question under discussion were obtained with two other antigens, namely SA and GIL azoproteins. Immune sera for SA gave precipitation with the homologous antigen and also with antigens containing only one of the residues, either succinanilic or phenylarsenic acid. S and A as well as SC sera reacted considerably better with the corre-

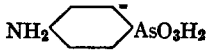
sponding than with SA (or SB) antigen. The tests are presented in Table I. The structure of the compounds used in these and the above experiments is shown in the formulae:



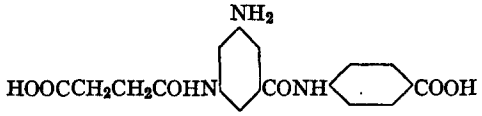
(3-amino, 5-succinylaminobenzoyl)-*p*-aminophenylarsenic acid (SA)



m-aminosuccinanilic acid (S)



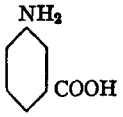
p-aminophenylarsenic acid (A)



(3-amino, 5-succinylaminobenzoyl)-*p*-aminobenzoic acid (SB)

p-aminobenzoic acid

3-amino, 5-carboxysuccinanilic acid (SC)



m-aminobenzoic acid

The presence of two distinct and unrelated sorts of antibodies could be shown by absorption with antigens (azostromata) that contained but one of the two determinant groups. Each of the "partial" antigens was seen to remove antibodies acting on its own determinant structure without affecting those for the other group. This held true even on repeated absorptions (Tables II*a* and II*b*).

Like absorption experiments, giving confirmatory results, were carried out on a second SA serum using A, SB and S antigens. After absorption with both antigens, A and S (or A and SB), the immune sera no longer gave any reaction with the homologous antigen SA.

Immune sera produced to an antigen containing the two amino acids, glycine and leucine, linked to one benzene ring also showed the presence of two distinct sorts of antibodies each directed towards one of the two determinants. Precipitin tests showing the cross reactions

TABLES II*a* AND *b*

SA immune serum was absorbed separately with SB and with A azostromata for 2 hours at room temperature, 8 mg. of azostromata being used for 3 cc. of undiluted immune serum. In each case a portion of the absorbed immune serum was treated similarly with a proportionate amount of the same azostromata; a third absorption was carried out in the same manner. For the tests 0.2 cc. of the absorbed fluids (designated in the tables as I, II and III) was mixed with 0.05 cc. of the test antigens (dilution 1:100 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).

TABLE II*a*

Repeated Absorptions with SB

	Test antigens		
	SA	SB	A
I	+±	0	++±
	+++	0	++++
II	+±	0	++±
	+++	0	++++
III	+±	0	++±
	++±	0	++++
Unabsorbed immune serum	+++	+++	++±
	++++	++++	++++

TABLE II*b*

Repeated Absorptions with A

	Test antigens		
	SA	SB	A
I	+++	+++	0
	++++	++++	0
II	+++	+++	0
	++++	++++	0
III	+++	+++	0
	+++±	+++±	0
Unabsorbed immune serum	+++	+++	++±
	++++	++++	++++

TABLE III

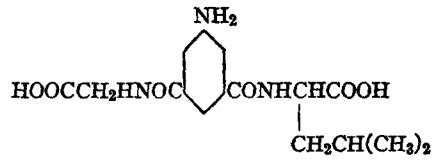
0.1 cc. of the immune sera was added to 0.2 cc. of the antigen solutions. Readings after 1 hour at room temperature.

Immune sera	Test antigens											
	GIL			G			L			GIPh		
	1:100	1:500	1:2500	1:100	1:500	1:2500	1:100	1:500	1:2500	1:100	1:500	1:2500
GIL	+±	++±	+±	±	+	±	∔	+±	±	+±	+±	tr.
G	0	∔	±	+±	++±	+	0	0	0	tr.	∔	±
L	±	+	±	0	f.tr.	f.tr.	tr.	++±	+			
G + L*	f.tr.	+	∔	∔	++	+	0	+±	∔			
G + L†	tr.	+	∔	∔	+±	+	tr.	++	+			

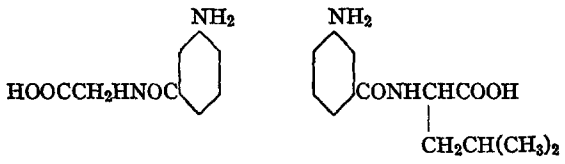
* Mixture of equal parts of G and L immune serum.

† Mixture of one part of G immune serum and two parts of L immune serum.

of GIL, G and L immune sera on the antigens with one or both determinants are presented in Table III, where in addition a comparison is made of GIL serum with mixtures of G and L immune sera. One example only is given for each kind of sera; the results with additional immune sera were in agreement.

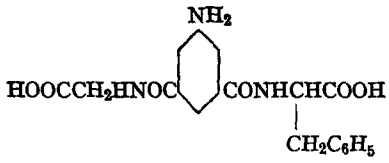


sym. aminoisophthalyl glycine-leucine (GIL)



m-aminobenzoyl glycine (G)

m-aminobenzoyl leucine (L)



sym. aminoisophthalyl glycine-phenylalanine (GIPh)

The separation of two sorts of antibodies in GIL serum by absorption is demonstrated in Tables IV*a* and *b*, there being stronger reactions on the leucine than on the glycine moiety. When the immune serum was treated with a mixture of G and L azostromata, the reaction with the "full" antigen became weak after two absorptions but there was still a faint reaction after the third absorption which may perhaps indicate the presence of a slight amount of a special antibody (Table V).

From the tests with mixed G and L immune sera (Table III) the inference may be drawn that the G and L antibodies found in the GIL serum are not the same as the antibodies present in G and L sera, for it

TABLES IV*a* AND *b*

GIL immune serum was absorbed separately with G and with L azostromata for 2 hours at room temperature, using 4.5 mg. of azostromata and 3 cc. of diluted immune serum (1:2). This procedure was repeated twice and tests were made with the fluids as in Tables II *a* and *b*.

TABLE IV*a*

Repeated Absorptions with G

	Test antigens		
	GIL	G	L
I	++±	0	++
	+++±	0	++±
II	++	0	+±
	+++±	0	++±
III	++	0	+±
	+++±	0	++±
Unabsorbed immune serum	+++	+	++
	+++±	+	++±

TABLE IV*b*

Repeated Absorptions with L

	Test antigens		
	GIL	G	L
I	++	+	0
	++±	+	0
II	+±	+	0
	++	+	0
III	+	+	0
	++	+	0
Unabsorbed immune serum	+++	+	0
	+++±	+	0

TABLE V

Repeated Absorption with G + L

GIL immune serum was absorbed with a mixture of G and L azostromata for 2 hours at room temperature, using 3 mg. azostromata of each kind for 3 cc. of immune serum (diluted 1:2 with saline). This procedure was repeated twice and the tests were made with the fluids (I, II and III) as above.

	Test antigens		
	GIL	G	L
I	+	0	0
	+±	0	tr.
II	tr.	0	0
	±	0	0
III	f.tr.	0	0
	tr.	0	0
Unabsorbed immune serum	+++	+	++
	+++±	+	+++±

appears that a mixture of the latter gives weaker precipitation with GIL antigen than do GIL immune sera whereas this is not the case in tests with G or L antigens. Inhibition experiments supported the above conclusion in so far as the antibodies in GIL serum reacting on G or L antigens were slightly better inhibited by GIL than by G or L; on the other hand, with G and L immune sera there was a difference in the opposite direction.

COMMENT

The experiments made with sera for the compounds containing both glycine and leucine (GIL), or succinic and phenylarsenic acid (SA) residues definitely establish the existence of cases in which discrete antibodies are formed that are individually directed towards separate determinant groups in one substance, even though it be of small molecular size and the determinant groups rather closely adjoining. The failure of repeated absorption with an antigen which contains only one of the two groupings, to effect a significant diminution of the antibody reacting with the other group, is proof for the lack of serological kinship between the two antibodies demonstrated. It is in contrast to the behavior of multiple but related antibodies developed through the stimulus of a single determinant structure, where continued absorption with a reacting heterologous antigen was often seen to exhaust the immune serum completely (4).[3] It may be noted that, in the substances here examined, the determinant groups are acid, namely the residues of succinic acid, phenylarsenic acid, glycine and leucine which have been shown to exert a prominent influence on specificity. Furthermore, comparative experiments with antisera produced by antigens with one acid group showed that the presence of a second group produced a qualitative change in the antibodies (*cf.* Morgan (3), (5)).

There were practically no antibodies in the sera examined which were not removable by absorption with both "partial" antigens. Also, antibodies that have two combining groups corresponding respectively to the two determinants of the substances investigated could not be demonstrated, and appear to be present in the GIL and SA immune sera, if at all, only in small quantities since, as just mentioned, exhaus-

[3] No attempts have been made in the present investigation to separate such fractions by absorption with suitable antigens.

tion with one partial antigen did not diminish the reaction for antigens containing the other determinant. Yet evidence, although calling for more extensive investigation, of the existence of such combining groups of different specificities in an antibody molecule[4] may perhaps be derived, as mentioned, from some observations on antibodies which appear to react at the same time with the protein and the hapten parts of azoproteins.

The results discussed, particularly the formation of antibodies against parts of the haptens studied, are connected with the issues debated lately of the univalence or multivalence of antibodies and the size of their combining areas. A relatively small specific site is indicated by the attachment of several antibody molecules to one molecule of antigen (*cf.* Haurowitz (8)), and may also explain why, remarkably, the various antibodies, both chemically and in their specificity, when employed as antigens, are rather similar to one another and, as well, to globulins present in normal sera. In this connection, however, it should be considered that the specificity of antibodies, or else their activity, may well depend on structures of a different character from those underlying antigen specificity; in fact, the specific properties of antibodies appear in general to be entirely destroyed by denaturation through heat and other agents, while denaturation causes alterations of comparatively lesser degree in the specific serological properties of protein antigens.

In the case of immune sera to peptides (16) or disaccharides (17), there has not been demonstrated so far the resolution of the whole immune body content into antibody fractions related to definite parts of the molecules such as these encountered in the present experiments. Future studies will be necessary to determine the structural features requisite for the production of antibodies severally directed towards different groups in one molecule and to ascertain to what extent such antibodies occur in immune sera prepared by means of natural antigens and how far they are responsible for cross reactions (*cf.* 4).[5] Of some significance, too, for the problem of immune bodies to natural antigens are the two instances reported, in which an appreciable amount of anti-

[4] See also Morgan (14), and Meyer (15).

[5] Since this paper was completed there has appeared a study by Marrack and Carpenter (18) in which this question is discussed with reference to the cross reactions of polysaccharides.

bodies was produced for one, only, of the two groups present though each had formerly been found to serve as a serological determinant.

The experiments were carried out with the assistance of Mr. B. Meier.

SUMMARY

Azoproteins have been prepared with azocomponents possessing two serologically active groups. On immunization with such antigens immune sera were obtained containing two separate, unrelated antibodies, each specific for one of the two groups and separable by absorption. In other cases one of the two structures was dominant, in that antibodies were formed only towards this and not towards the other grouping. The specificity of the antibodies was in general found to be influenced to some extent by the presence of a second group in the antigen. The relevancy of these observations for antibodies directed against natural antigens has been noted.

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