Psychiatric Institute

SEROLOGICAL DIFFERENTIATION OF STERIC ISOMERS.

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In previous work[1] the conclusion was arrived at that serological specificity depends on the chemical constitution of the reacting groups in general and probably also on their spatial configuration. The latter assumption had been made on account of the reactions observed with antigens containing aromatic compounds substituted in various positions of the benzene nucleus. The experiments dealt with in the present paper were carried out in order to secure conclusive evidence for this conception. Thus we endeavored to determine whether optical isomers can be differentiated by means of serum reactions.

The substances chosen for this purpose were *l*- and *d*-phenyl (para-aminobenzoylamino) acetic acid. These compounds had been used by Ingersoll and Adams[2] in an investigation on the affinity to fibers, of optically isomeric dyes. By diazotization and coupling to dimethylaniline they obtained two isomeric dyes which were absorbed by wool in different amounts; when β-naphtol was used in place of dimethylaniline the dyes were absorbed equally well.

The preparation of the phenyl (para-aminobenzoylamino) acetic acid was carried out in the main according to the directions of Ingersoll and Adams. Phenylaminocyanide made by the interaction of benzaldehyde, sodium cyanide, and ammonium chloride in water solution, was hydrolyzed to phenylaminoacetic acid.[3] The *d*- *l*- phenylaminoacetic acid was resolved into the levo and dextro product by means of fractional crystallization of the *d*-camphor sulfonate and *l*-camphor sulfonate respectively. In the recrystallization of the *l*-amino acid *d*-camphor

[1] A review of the subject can be found in The chemical aspects of immunity, by H. Gideon Wells, American Chemical Society, Monograph Series, New York, 1925, 77.

[2] Ingersoll, A. W., and Adams, R., *J. Am. Chem. Soc.*, 1922, xliv, 2930; 1925, xlvii, 1169.

[3] *Cf.* Marvel, C. S., and Noyes, W. A., *J. Am. Chem. Soc.*, 1920, xlii, 2264.

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sulfonate it was found of advantage to filter off the crystals when the solution had cooled to about 45°. Few recrystallizations were sufficient to obtain a pure product. For the isolation of the *d*-amino acid, inactive camphor sulfonic acid was used. The *d*-amino acid *l*-camphor sulfonate was purified by several recrystallizations.

Both forms of phenylaminoacetic acid were condensed with para-nitrobenzoylchloride to form *l*- and *d*-phenyl (*p*-nitrobenzoylamino) acetic acid $HO_2CC(C_6H_5)$-$HNHCOC_6H_4NO_2$. For recrystallization of this substance thirty times its weight of a boiling mixture of one part of alcohol and two parts of water was used. Crystallization was allowed to take place while stirring. The melting points agreed with those given by the authors named. The nitro compounds were reduced to *l*- and *d*-phenyl (para-aminobenzoylamino) acetic acid by means of ferrous sulfate and ammonia and the final products were recrystallized from water. The levo form had the melting point 168–169°, 0.400 gm. dissolved and made up to 5 cc. with normal HCl gave at 20° a rotation of −14.97 in a 2 dm. tube, with sodium light. For this concentration Ingersoll and Adams give a rotation of −15.00 and the melting point 168–169°. The dextro form had a melting point of 168–169°; 0.400 gm. dissolved and brought up to a volume of 5 cc. with normal HCl at 20° gave a rotation of +14.98 in a 2 dm. tube with sodium light, in accordance with the data of Ingersoll and Adams.

d-l-Phenyl (para-aminobenzoylamino) acetic acid was prepared in the manner described by the authors quoted, from the inactive phenylaminoacetic acid. Melting point 152°.

The levo- and dextro-phenyl (para-aminobenzoylamino) acetic acids will for the sake of convenience be designated as *l*- and *d*-acid. The azoproteins prepared from these amino acids by diazotization and coupling to proteins will be referred to as *l*- and *d*-antigens and likewise the sera obtained by immunization with the azoproteins as *l*- and *d*-immune sera, respectively; *i* denotes the inactive preparations.

Preparation of the l- and d-Antigens for Immunization.—The *l*- and *d*-acids were coupled to proteins in the following manner: 17.12 gm. of the optically active compound were dissolved in 200 cc. water and 25 cc. of 7 normal HCl and diazotized with the required amount of sodium nitrite at a temperature of 0–5°C. with starch iodide paper as indicator; the diazo solution was diluted with ice water to a volume of 500 cc. and poured into a mixture of 500 cc. of horse serum and 500 normal sodium carbonate; coupling was allowed to take place for 10 minutes at 0–5°. By acidification with hydrochloric acid the azoprotein was precipitated and after filtration it was dissolved in a small volume of water by addition of a little normal sodium carbonate. It was reprecipitated from this solution with a large quantity of alcohol and enough hydrochloric acid to flocculate the material. The precipitate was filtered, again treated in the same manner, and the azoprotein was freed from alcohol by redissolving in water and sodium carbonate, and precipitation with hydrochloric acid. It was finally dissolved and brought to a volume of 950 cc. using just enough sodium carbonate to give neutral reaction to litmus

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TABLE I.

To 0.2 cc. of the diluted antigen were added 3 capillary drops of *l*-immune serum.

Readings taken after:	*l*-Antigen prepared with chicken serum Dilution 1:				*d*-Antigen prepared with chicken serum Dilution 1:			
	20	100	500	2500	20	100	500	2500
2 hrs. at room temperature	++	+±	+	tr.	+±	0	0	0
Night in ice box	+++	++	++	+±	++	±	f.tr.	0

TABLE II.

To 0.2 cc. of the diluted antigen were added 4 capillary drops of *d*-immune serum.

Readings taken after:	*l*-Antigen made with chicken serum Dilution 1:				*d*-Antigen made with chicken serum Dilution 1:			
	20	100	500	2500	20	100	500	2500
2 hrs. at room temperature	++	0	0	0	+±	+±	+±	±
Night in ice box	+++	±	0	0	+++	+++	++	+

TABLE III, *a*.

To 0.2 cc. of the diluted antigens were added 3 capillary drops of *l*-immune serum.

Readings taken after:	*l*-Antigen made with chicken serum Dilution 1:		*d*-Antigen made with chicken serum Dilution 1:		*i*-Antigen made with chicken serum Dilution 1:	
	100	500	100	500	100	500
2 hrs. at room temperature	+	+	0	0	+	±
Night in ice box	++±	++	±	0	++±	+±

TABLE III, *b*.

To 0.2 cc. of the diluted antigens were added 2 capillary drops of *d*-immune serum.

Readings taken after:	*l*-Antigen made with chicken serum Dilution 1:		*d*-Antigen made with chicken serum Dilution 1:		*i*-Antigen made with chicken serum Dilution 1:	
	100	500	100	500	100	500
2 hrs. at room temperature	f.tr.	0	+	+	+	±
Night in ice box	+	0	++	++	++	++

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and the necessary amount of a salt solution to make the ultimate salt concentration approximately 1 per cent. 50 cc. of a 5 per cent phenol solution was added.

TABLE IV, *a*.

0.2 cc. of *l*-antigen (diluted 1:500) prepared with chicken serum was mixed with 0.05 cc. of a neutral solution containing 1 millimol of the substances indicated, in 10 cc. To this 3 capillary drops of *l*-immune serum were added. The control tube contains only immune serum and antigen.

Readings taken after:	1	2	3	4	5	6	7	8	*l*-Acid	*d*-Acid	*i*-Acid	Control
3 hrs. at room temperature	±	+	+	+	±	+	±	+	0	±	f.tr.	+±
Night in ice box	+±	+±	++	++	++	+±	+±	++	tr.	+±	+	++

TABLE IV, *b*.

0.2 cc. of *d*-antigen (diluted 1:500) prepared with chicken serum was mixed with 0.05 cc. of a neutral solution containing 1 millimol of the substances indicated in the first line of the table in 10 cc. To this mixture 3 capillary drops of *d*-immune serum were added.

Readings taken after:	1	2	3	4	5	6	7	8	*l*-Acid	*d*-Acid	*i*-Acid	Control
3 hrs. at room temperature	+	+	+±	+±	+	+	+	+±	±	0	0	+±
Night in ice box	++	++	++	++	++	++	+±	++±	+±	0	+	++±

TABLE IV, *c*.

The following tests were made like those given in Tables IV,*a* and IV,*b*, but 0.05 cc. of solution containing 0.5 millimol of the *d*-, *l*-, and *i*-acids was added.

Readings taken after:	*l*-Antigen and 3 capillary drops *l*-immune serum, with addition of:				*d*-Antigen and 3 capillary drops *d*-immune serum, with addition of:			
	l-Acid	*d*-Acid	*i*-Acid	Control	*l*-Acid	*d*-Acid	*i*-Acid	Control
3 hrs. at room temperature	0	±	0	+	+	0	±	+±
Night in ice box	±	++	+±	++	++	tr.	+	++±

Immunization.—Two series of seven rabbits each were injected with 15 cc. of the *l*- and *d*-antigens at weekly intervals. In each of the two series one serum of medium strength was produced and in addition some weakly reacting sera.

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Antigens for the Tests.—These were prepared in the same way as the antigens for immunization; as protein component chicken serum was used. After coupling, the azoprotein was precipitated with acid, washed with water, and brought into solution by means of sodium carbonate. The quantity of antigen in the solution was determined by precipitation with alcohol and weighing of the dried substance. The dilutions 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), ±, +, +±, etc.

The immune sera obtained were tested against the *l*-, *d*-, and *i*-antigens with the results given in Tables I, II, and III *a*, *b*.

In addition to the tests presented others were made in order to determine the inhibiting effect on the precipitin reaction,[4] by the *l*-, *d*-, and *i*-acids and other substances, namely; (1) benzoic acid; (2) para-aminobenzoic acid; (3) sulfanilic acid; (4) acetic acid; (5) phenylacetic acid; (6) hippuric acid; (7) phenylglycocoll; (8) phenylalanine (Tables IV,*a*, IV,*b*, IV,*c*).

DISCUSSION AND SUMMARY.

The tests presented in Tables I, II, and III show that only in the higher concentration cross reactions do take place, and that there is definite specificity of the two sorts of immune sera for the homologous antigens. Thus it is easy to differentiate the *l*- and *d*-antigens in dilutions 1:100 and upwards. The occurrence of cross reactions can readily be ascribed to the fact that the *l*- and *d*-acids present in the two antigens are identical in every respect but the position of the groups connected with the asymetric carbon atom. The *i*-antigen reacts with both sorts of immune sera as could be expected since it must consist of a mixture of equal parts of *l*- and *d*-antigen.

The reactions of the *i*-antigen appear to be only slightly weaker than those of the homologous ones owing to the fact that the intensity of the reactions diminishes but slowly with increasing dilution of the antigens. It is also to be considered that small differences cannot be judged very accurately.

Tests with two *l*- and four *d*-immune sera[5] not recorded in the tables confirmed the results already discussed.

[4] *Cf.* Landsteiner, K., *Biochem. Z.*, 1920, civ, 280.

[5] Two of these, of medium strength, were obtained from an additional immunization experiment.

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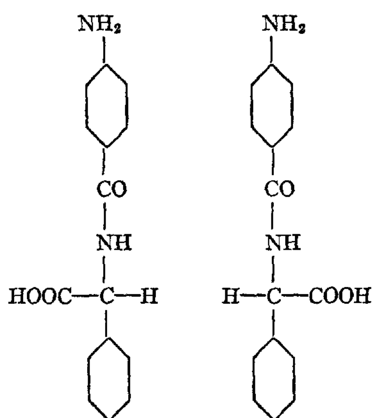
Considering that ferments are known to be adapted ordinarily to one type of steric isomers it may be worth noting that antibodies were formed by the same species of animals for optical antipodes.

From the results summarized in Tables IV,*a* and IV,*b* one sees that the *l*- and *d*-immune sera also differentiate clearly between the *l*- and *d*-acids when they are not diazotized and not combined with protein. The *l*-acid inhibits much more the precipitation of the *l*-antigen by the homologous immune serum than the *d*-acid and the converse effect occurs if the inhibiting action is tested on the precipitation of *d*-antigen by *d*-immune serum. The inactive phenyl (para-aminobenzoylamino) acetic acid behaved in such tests as a mixture of *l*- and *d*-acids, *i.e.*, it acted markedly in both cases, more than the heterologous and less than the homologous acid.

The experiments reported bring a definite proof for the view that the steric configuration of antigenic groups is one of the factors determining serological specificity. In the particular case under consideration the mere difference in the position of H and COOH as indicated in the following formulas sufficed to alter the reactivity.



The fact that steric isomers are acted upon selectively by immune sera may be supposed to play a significant part in the serological specificity of carbohydrates such as those discovered in bacterial antigens.

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