ON THE SEROLOGICAL SPECIFICITY OF PEPTIDES. II

By K. LANDSTEINER, M.D., and J. VAN DER SCHEER

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

(Received for publication, March 9, 1934)

The results of serological tests with azoproteins prepared from peptides of known structure, namely glycyl-glycine, glycyl-leucine, leucyl-glycine and leucyl-leucine, were reported in a previous communication (1). By means of precipitin and inhibition tests it was found that these peptides were serologically distinct and that their specificity was determined primarily by the terminal amino acid and to a lesser extent by the second amino acid. These experiments were taken up again and extended to other peptides.

Preparation of Peptides and Their Nitrobenzoyl and Aminobenzoyl Derivatives

The following peptides were prepared according to the methods given in the papers referred to: diglycyl-glycine, triglycyl-glycine, tetraglycyl-glycine, di-*d,l*-leucyl-glycyl-glycine (2); diglycyl-*d,l*-leucine, triglycyl-*d,l*-leucine (3); glycyl-*d,l*-leucyl-glycine (4); *d,l*-leucyl-glycyl-glycine (5); *d,l*-leucyl-triglycyl-glycine (6).

Chloracetyl-d,l-Leucyl-Glycyl-Glycine.—30 gm. of *d,l*-leucyl-glycyl-glycine were dissolved in 124 cc. of N sodium hydroxide and the solution was cooled to −5°C. 28 gm. of chloracetylchloride and 76 cc. of 5 N sodium hydroxide were added alternately in 5 equal portions with good cooling and vigorous shaking. The solution was made strongly acid to Congo red by addition of 5 N hydrochloric acid and extracted several times with ethyl acetate containing 10 per cent of alcohol. The ethyl acetate solution was dried with anhydrous sodium sulfate and evaporated. The remaining oil crystallized upon stirring with ether. Yield 31 gm. It was recrystallized by dissolving in 20 parts of ethyl acetate and allowing the solution to stand in the ice box for 1 day. Yield 20 gm. Microcrystalline, m.p. 149–150°.[1] Analysis: After drying at 100° *in vacuo* over $CaCl_2$; calculated for $C_{12}H_{20}O_5N_3Cl$: N 13.07, found 13.12; Cl 11.02, found 10.90.

[1] The melting points were made with the analyzed substances but were not checked by further purification.

Glycyl-d,l-Leucyl-Glycyl-Glycine.—A solution of 1 part of chloracetyl-*d,l*-leucyl-glycyl-glycine in 10 parts of ammonium hydroxide (28 per cent) was heated in a sealed tube at 100°C. for 1½ hours. The solution was evaporated to dryness *in vacuo*, the residue dissolved in water, treated with a solution of silver sulfate to remove Cl^-, and subsequently with hydrogen sulfide. After removal of silver sulfide and evaporation *in vacuo* to a small volume just enough barium hydroxide was added to free the solution from sulfates. The solution was evaporated to dryness *in vacuo*, the material dissolved in a very small amount of water, 10 volumes of absolute alcohol and 20 volumes of ether were added and the sticky precipitate was rubbed with a mixture of 1 part of absolute alcohol and 2 parts of dry ether until it became granular. Yield 2.4 gm. from 3 gm. of the chloracetyl compound. Amorphous, decomposes when heated above 120°. Analysis: After drying at 100° *in vacuo* over $CaCl_2$; calculated for $C_{12}H_{22}O_5N_4$: N 18.51, found 18.40.

Chloracetyl-Glycyl-d,l-Leucyl-Glycyl-Glycine.—This substance was prepared from glycyl-*d,l*-leucyl-glycyl-glycine as described for chloracetyl-*d,l*-leucyl-glycyl-glycine. The ethyl acetate solution was evaporated *in vacuo* and absolute alcohol was added. A white insoluble residue was separated from the colored alcoholic solution and recrystallized by dissolving in 40 parts of 95 per cent alcohol and allowing the solution to stand in the ice box overnight. Yield 6 gm. from 12 gm. of peptide. Small platelets, m.p. 195–196°C. Analysis: After drying at 100° *in vacuo* over $CaCl_2$; calculated for $C_{14}H_{23}O_6N_4Cl$: N 14.80, found 14.59; Cl 9.37, found 9.27.

Diglycyl-d,l-Leucyl-Glycyl-Glycine.—1 part of chloracetyl-glycyl-*d,l*-leucyl-glycyl-glycine was heated in a sealed tube with 10 parts of ammonium hydroxide (28 per cent) for 1½ hours at 100°C. The solution was evaporated *in vacuo*, the peptide redissolved in 2 parts of water and 20 volumes of absolute alcohol were added. After standing in the ice box the precipitated peptide was centrifuged off, repurified in the same manner and washed with alcohol. Yield 3.5 gm. from 6 gm. chloracetyl compound. Not distinctly crystalline, decomposes above 215°. Analysis: After drying at 100°C. *in vacuo* over $CaCl_2$, calculated for $C_{14}H_{25}O_6N_5$: N 19.49, found 19.15.

α-Bromisocaproyl-Dileucyl-Glycyl-Glycine (Inactive).—This substance was prepared from dileucyl-glycyl-glycine (inactive) and *d,l*-α-bromisocaproylchloride according to the method described by Fischer for the preparation of α-bromisocaproyl-leucyl-glycyl-glycine (2). After completion of the acetylation the solution was acidified with hydrochloric acid, and the precipitate washed with petrol ether. It was recrystallized from 25 parts of 40 per cent alcohol. Yield 10 gm. from 10 gm. of peptide. Microscopic needles, m.p. 192–193° Analysis: After drying at 100°C. *in vacuo* over $CaCl_2$; calculated for $C_{22}H_{39}O_6N_4Br$: N 10.50, found 10.62.

Trileucyl-Glycyl-Glycine (Inactive).—10 gm. of α-bromisocaproyl-dileucyl-glycyl-glycine (inactive) was heated with 50 cc. of ammonium hydroxide (28 per cent) in a sealed tube at 100° for 1½ hours. After evaporation on the steam bath the sub-

stance was stirred with absolute alcohol, the alcohol evaporated and the peptide suspended in 100 cc. absolute alcohol and kept in the ice box overnight. It was extracted a second time with absolute alcohol. Yield 5.7 gm. Amorphous, turns dark above 210°. Analysis: After drying at 100°C. *in vacuo* over $CaCl_2$; calculated for $C_{22}H_{41}O_6N_5$: N 14.87, found 14.60.

Chloracetyl-Triglycyl-d,l-Leucine.—This substance was prepared from trigylcyl-*d,l*-leucine and chloracetyl chloride in the usual way (3). The chloracetyl peptide separated from the reaction mixture upon acidification and was recrystallized from 5 parts of water. Yield 18 gm. from 30 gm. of peptide. Amorphous, m.p. 185–187°. Analysis: After drying at 100° *in vacuo* over $CaCl_2$; calculated for $C_{14}H_{23}O_6N_4Cl$: N 14.80, found 14.74.

Tetraglycyl-d,l-Leucine.—A solution of 10 gm. of chloracetyl-triglycyl-*d,l*-leucine in 250 cc. of 28 per cent ammonium hydroxide was kept at 37° for 3 days. Ammonia was removed by evaporation to dryness *in vacuo* and the substance was purified by the silver sulfate method. The solution of the peptide was evaporated to dryness *in vacuo*, the substance was dissolved in 15 cc. of water, 300 cc. of absolute alcohol were added and subsequently 3 volumes of dry ether to precipitate the peptide. Yield 4 gm. from 6 gm. of chloracetyl compound. The substance proved to be impure (calculated for $C_{14}H_{25}O_6N_5$: N 19.49, found 18.27). It was converted into the nitrobenzoyl derivative which crystallized easily and gave a correct analytical value for N.

The preparation of the *p*-nitrobenzoyl and *p*-aminobenzoyl derivatives of the following amino acids and peptides has been described previously (1): glycine, *d,l*-leucine, glycyl-glycine, glycyl-*d,l*-leucine, *d,l*-leucyl-glycine, *d,l*-leucyl-*d,l*-leucine A.

The nitrobenzoylation of the other peptides was in general made in the following manner.

A solution of 0.1 mol of the peptide in 600 cc. of 10 per cent sodium bicarbonate was mixed with 300 cc. of chloroform and a total of 55.5 gm. of finely ground *p*-nitrobenzoyl chloride (0.3 mol) was added in 5 equal portions over a period of 1½ hours with vigorous shaking at room temperature. Ether was added and the aqueous solution, after separation from the ether-chloroform mixture, was filtered and made acid to Congo red by addition of hydrochloric acid. The precipitate was filtered off, washed with water and dried at 45° *in vacuo*. (The precipitate of the crude nitrobenzoyl derivative separated either immediately or after a longer time, up to 1 or 2 days, at low temperature). The substances were finely ground and extracted several times with boiling ether to remove *p*-nitrobenzoic acid. They were then dissolved in water by addition of alkali, reprecipitated with acid and after drying again extracted with ether.

The *p*-aminobenzoyl peptides were obtained by reduction of the *p*-nitrobenzoyl peptides as follows:

The nitrobenzoyl peptides, dissolved in about 3 parts of water by addition of a slight excess of ammonium hydroxide and heating, if necessary, were added to a

hot solution of ferrous sulfate, 7 aq. (6.5 mols for each mol of nitrobenzoyl peptide) in 2.5 parts of water. A 28 per cent ammonia solution (10 cc. for each 12 gm. of ferrous sulfate, 7 aq.) was added in 5 equal portions over a period of 10 minutes, shaking well with each addition. After heating on the steam bath for 15 minutes the ferric hydroxide was removed by filtration and the solution evaporated *in vacuo* to a small volume. The subsequent procedure varied somewhat for the different substances.

p-Nitrobenzoyl-Diglycyl-Glycine.—Recrystallized by dissolving in 170 parts of boiling 70 per cent alcohol and allowing the solution to stand in the ice box overnight. Yield 9 gm. from 6 gm. of peptide. Large needles, turns dark above 245°. Analysis:[2] Calculated for $C_{13}H_{14}O_7N_4$: N 16.57, found 16.45.

p-Aminobenzoyl-Diglycyl-Glycine.—The concentrated solution was made weakly acid to Congo red with hydrochloric acid and kept in the ice box overnight. The precipitate was recrystallized from 25 parts of water. Yield 3.2 gm. from 4 gm. of nitro compound. Rosettes of small needles, turns dark above 245°. Analysis: Calculated for $C_{13}H_{16}O_5N_4$: N 18.19, found 17.91.

p-Nitrobenzoyl-Diglycyl-d,l-Leucine.—Recrystallized from 40 parts of 30 per cent alcohol. Yield 6 gm. from 6 gm. of the peptide. Microscopic platelets, m.p. 175–176°. Analysis: Calculated for $C_{17}H_{22}O_7N_4$: N 14.21, found 14.11.

p-Aminobenzoyl-Diglycyl-d,l-Leucine.—The concentrated solution was made weakly acid to Congo red and kept in the ice box overnight. The precipitate was recrystallized from 25 parts of water. Yield 4 gm. from 6 gm. of nitrobenzoyl compound. Microscopic needles, m.p. 176–177°. Analysis: Calculated for $C_{17}H_{24}O_5N_4$: N 15.38, found 15.34.

p-Nitrobenzoyl-Glycyl-d,l-Leucyl-Glycine.—Recrystallized from 20 parts of 30 per cent alcohol. Yield 9 gm. from 10 gm. of peptide. Rosettes of microscopic needles, m.p. 204–205°. Analysis: Calculated for $C_{17}H_{22}O_7N_4$: N 14.21, found 14.13.

p-Aminobenzoyl-Glycyl-d,l-Leucyl-Glycine.—To the concentrated solution 10 volumes of alcohol were added, ammonium sulfate was filtered off, the filtrate evaporated to dryness *in vacuo*, the residue dissolved in a small amount of water and after addition of hydrochloric acid to weak acidity to Congo red, the solution was concentrated in a vacuum desiccator until the aminobenzoyl peptide separated. It was recrystallized from 4 parts of water. Yield 3.4 gm. from 4.8 gm. of nitrobenzoyl compound. Microscopic platelets, m.p. 179–181°. Analysis: Calculated for $C_{17}H_{24}O_5N_4$: N 15.38, found 14.91.

p-Nitrobenzoyl-d,l-Leucyl-Glycyl-Glycine.—Prepared by nitrobenzoylation as described by Abderhalden, Dinerstein and Genes (7). M.p. 167–168°. Analysis: Calculated for $C_{17}H_{22}O_7N_4$: N 14.24, found 14.04.

p-Aminobenzoyl-d,l-Leucyl-Glycyl-Glycine.—After the reduction of nitrobenzoyl-

[2] For analysis the substances were dried at 100° *in vacuo* over $CaCl_2$ unless differently stated.

d,l-leucyl-glycyl-glycine the solution was evaporated *in vacuo* to a small volume, 10 volumes of alcohol were added, ammonium sulfate filtered off and the alcoholic solution evaporated to dryness *in vacuo*. The substance was dissolved in a small amount of alcohol and precipitated by addition of ether. After drying, it was dissolved in 2 parts of water and enough 5 N hydrochloric acid was added to make the solution weakly acid to Congo red. After being kept for 2 days at room temperature, the precipitate was filtered off and recrystallized from 5 volumes of water, using norit for decolorizing. Crystallization was allowed to take place for 2 days at room temperature. Yield 4.2 gm. from 8.4 gm. of nitrobenzoyl compound. Irregular platelets, m.p. 179–180°. Analysis: Calculated for $C_{17}H_{24}O_5N_4$: N 15.38, found 15.31.

p-Nitrobenzoyl-Triglycyl-Glycine.—Recrystallized from 20 parts of 50 per cent alcohol. Yield 11 gm. from 10 gm. of peptide. Microscopic needles, decomposes at about 230°. Analysis: Calculated for $C_{15}H_{17}O_8O_5$: N 17.72, found 17.49.

p-Aminobenzoyl-Triglycyl-Glycine.—The concentrated solution was made weakly acid to Congo red. The substance was reprecipitated from an alkaline solution by addition of acid, washed with water, 50 per cent alcohol and finally with absolute alcohol and ether. Upon cooling a hot aqueous solution it separated in the form of granules. Yield 4.2 gm. from 6 gm. of nitro compound. Decomposed without melting when heated above 250°. Analysis: Calculated for $C_{15}H_{19}O_6N_5$: N 19.18, found 18.83.

p-Nitrobenzoyl-Triglycyl-d,l-Leucine.—Recrystallized from 10 parts of 30 per cent alcohol. Yield 6.2 gm. from 6 gm. of peptide. Microscopic needles, begins to sinter above 95°, decomposes at about 220°. Analysis: After drying at 80° *in vacuo* over H_2SO_4, calculated for $C_{19}H_{25}O_8N_5$: N 15.52, found 15.41.

p-Aminobenzoyl-Triglycyl-d,l-Leucine.—The concentrated solution was made weakly acid to Congo and kept at room temperature overnight. The precipitate was redissolved in 5 parts of water and crystallization was allowed to take place in the ice box. Yield 1.9 gm. from 2.7 gm. of nitrobenzoyl peptide. Microscopic needles, m.p. 176–178°. Analysis: Calculated for $C_{19}H_{27}O_6N_5$: N 16.65, found 16.28.

p-Nitrobenzoyl-Glycyl-d,l-Leucyl-Glycyl-Glycine.—Nitrobenzoylation was carried out in a freezing mixture as in the case of *p*-nitrobenzoyl-*d,l*-leucyl-glycyl-glycine. The nitrobenzoyl compound was precipitated from the cold alkaline solution by acidification with hydrochloric acid. It was freed from *p*-nitrobenzoic acid by repeated extractions with ether following the general procedure described above, dissolved in 40 parts of boiling water and allowed to crystallize in the ice box. Yield 6 gm. from 10 gm. of peptide. Clumps of microscopic needles, decomposes above 220°. Analysis: Calculated for $C_{19}H_{25}O_8N_5$: N 15.52, found 15.44.

p-Nitrobenzoyl-Tetraglycyl-Glycine.—Recrystallized from 200 parts of 50 per cent alcohol. Yield 9 gm. from 10 gm. of peptide. Rosettes of microscopic needles, decomposes above 240°. Analysis: Calculated for $C_{17}H_{20}O_9N_6$: N 18.59, found 18.38.

p-Aminobenzoyl-Tetraglycyl-Glycine.—Redissolved with alkali and precipitated with acid, washed with water, 50 per cent alcohol and finally with absolute alcohol and ether. Yield 5 gm. from 7 gm. of nitrobenzoyl compound. Amorphous, decomposes above 270°. Analysis: Calculated for $C_{17}H_{22}O_7N_6$: N 19.92, found 19.69.

p-Nitrobenzoyl-Tetraglycyl-Leucine.—This substance was prepared by nitrobenzoylation of impure tetraglycyl-leucine in the usual way in a solution of 10 per cent sodium bicarbonate and adding the *p*-nitrobenzoyl chloride dissolved in benzene. The alkaline solution was made weakly acid to Congo red and kept in the ice box for 1 hour. After removal of the precipitate the solution was made strongly acid to Congo red by the addition of hydrochloric acid and was kept in the ice box for 2 days. The precipitate was filtered, washed with cold water, dried and freed from *p*-nitrobenzoic acid by extractions with ether. It was recrystallized from 20 parts of water. Yield 4.2 gm. from 7.2 gm. of peptide. Microcrystalline, no definite crystal form, m.p. 191–193°. Analysis: Calculated for $C_{21}H_{28}O_9N_6$: N 16.54, found 16.44.

p-Aminobenzoyl-Tetraglycyl-d, l-Leucine.—When the solution was concentrated after the reduction and made weakly acid to Congo red, the substance separated as an oil which solidified upon cooling. It was freed from ammonium sulfate by dissolving in hot alcohol and the alcoholic solution was evaporated to dryness *in vacuo*. The substance was redissolved in 2 parts of water and kept in the ice box until some brownish material separated and the colorless solution after filtration was evaporated to dryness in a vacuum desiccator. Yield 0.8 gm. from 2 gm. of nitrobenzoyl peptide. Softens at 165°. Analysis: Calculated for $C_{21}H_{30}O_7N_6$: N 17.58, found 17.63.

p-Nitrobenzoyl-Diglycyl-d,l-Leucyl-Glycyl-Glycine.—Nitrobenzoylation was carried out as for the preparation of *p*-nitrobenzoyl-glycyl-*d,l*-leucyl-glycyl-glycine. The substance was precipitated from the alkaline solution by acidification, dried and freed from *p*-nitrobenzoic acid by extractions with ether. It was dissolved in 15 parts of boiling water and the precipitate was filtered after the solution had been kept overnight in the ice box. Yield 7 gm. from 10 gm. of peptide. Amorphous, m.p. 143–145°. Analysis: Calculated for $C_{21}H_{28}O_9N_6$: N 16.54, found 16.30.

p-Aminobenzoyl-Diglycyl-d,l-Leucyl-Glycyl-Glycine.—The concentrated solution obtained after reduction of the nitrobenzoyl peptide was freed from ammonium sulfate by addition of alcohol, and the substance after evaporation of the alcohol was redissolved in boiling absolute alcohol and precipitated from the filtered alcoholic solution by addition of 5 volumes of ether and dried. To 2 gm. dissolved in 1 cc. water 1 cc. of glacial acetic acid was added and the aminobenzoyl peptide was precipitated from the solution by the addition of acetone. Yield 1.8 gm. from 3.8 gm. of nitrobenzoyl compound. Softens at about 120°. Analysis: After drying at 60° *in vacuo* over H_2SO_4, calculated for $C_{21}H_{30}O_7N_6$: N 17.58, found 17.71.

p-Nitrobenzoyl-d,l-Leucyl-Triglycyl-Glycine.—Recrystallized from 40 parts of 30 per cent alcohol. Yield 9.5 gm. from 10 gm. of peptide. Rosettes of microscopic needles, decomposes above 225°. Analysis: After drying at 50° *in vacuo* over H_2SO_4, calculated for $C_{21}H_{28}O_9N_6$: N 16.54, found 16.31.

p-Aminobenzoyl-d,l-Leucyl-Triglycyl-Glycine.—The solution of the ammonium salt of the aminobenzoyl peptide was worked up as in the case of *p*-aminobenzoyl-diglycyl-*d,l*-leucyl-glycyl-glycine, and the substance was precipitated from a solution in 95 per cent alcohol by addition of ether. After drying, 2 gm. were dissolved in 1 cc. water, 1 cc. of glacial acetic acid was added and the amino compound was precipitated by the addition of acetone. It was redissolved in 10 cc. of absolute alcohol, the solution was freed from a small amount of insoluble material and the substance precipitated by addition of 70 cc. of acetone. Yield 1.3 gm. from 3 gm. of nitrobenzoyl compound. Analysis: Calculated for $C_{21}H_{30}O_7N_6$: N 17.58, found 17.81.

p-Nitrobenzoyl-Trileucyl-Glycyl-Glycine (Inactive).—Nitrobenzoylation was carried out as in the case of *p*-nitrobenzoyl-glycyl-*d,l*-leucyl-glycyl-glycine. The substance was precipitated from the solution by acidification and extracted with ether. Recrystallized from 40 parts of 70 per cent alcohol. Yield 8.5 gm. from 10 gm. of peptide. Rosettes of microscopic needles, m.p. 222–224°, with darkening. Analysis: Calculated for $C_{29}H_{44}O_9N_6$: N 13.55, found 13.62.

p-Aminobenzoyl-Trileucyl-Glycyl-Glycine (Inactive).—The nitrobenzoyl peptide was converted into the amino compound by dissolving 2.5 gm. in 70 cc. of 60 per cent alcohol containing 1.5 cc. concentrated ammonium hydroxide and reducing with ferrous sulfate, as described above. After heating on the steam bath the solution was filtered, the ferric hydroxide precipitate washed with hot 50 per cent alcohol and the combined solutions concentrated *in vacuo* to a small volume. The amorphous substance was filtered off after addition of sufficient hydrochloric acid to make the liquid weakly acid to Congo red. It was dissolved in a small amount of water by addition of alkali, the solution was made acid to Congo red with hydrochloric acid, some acid-insoluble material was removed by centrifugalization and the amino compound was reprecipitated from the solution with the required amount of alkali. Yield 1.2 gm. from 2.4 gm. of nitrobenzoyl compound. Amorphous. Softens at 165°. Analysis: Calculated for $C_{29}H_{46}O_7N_6$: N 14.24, found 14.20.

p-Nitrobenzoyl-Glutathione.—To a solution of 2.5 gm. of glutathione in 30 cc. of water 6 gm. of sodium bicarbonate were added and subsequently 4.3 gm. of *p*-nitrobenzoyl chloride dissolved in benzene in 5 equal portions. The mixture was shaken vigorously for 1½ hours. After filtration the aqueous solution was made acid to Congo red with hydrochloric acid, kept in the ice box overnight and the substance after drying was freed from *p*-nitrobenzoic acid by extractions with ether. Yield 2 gm. Amorphous. Softens at 140°. Analysis: After drying at 50° *in vacuo* over $CaCl_2$; calculated for the reduced form, $C_{17}H_{20}O_9N_4S$: S 7.02, found 7.03.

p-Aminobenzoyl-Glutathione.—After reduction of the nitro compound the solution was concentrated *in vacuo* to a small volume. Upon addition of hydrochloric acid a sticky yellow precipitate was formed which was separated from the liquid and redissolved in water by addition of sodium hydroxide. The amino compound was precipitated from the neutral solution in the form of a copper salt by addition of

copper sulfate. After filtration and washing with water the copper salt was suspended in water and decomposed with hydrogen sulfide. After filtration the copper sulfide was extracted with hot alcohol. The aqueous solution and the alcoholic extract were joined and evaporated to dryness, the substance was dissolved in alcohol and precipitated with 6 volumes of ether. Yield 1.2 gm. from 2.7 gm. of nitrobenzoyl compound. Amorphous, decomposes without melting above 105°. Analysis: After drying at 80° *in vacuo* over H_2SO_4; calculated for the reduced form, $C_{17}H_{22}O_7N_4S$: N 13.14, S 7.51; found N 12.92, S 7.58.

Serological Tests

Preparation of Antigens.—The antigens were made as described previously (1). The azoproteins used for immunization were purified by repeated washings with saline, omitting the precipitation with alcohol. Some of the antigens, namely those used for the production of the immune sera GL No. 2 and LG No. 2 were prepared with one-half of the indicated quantity of the aminobenzoyl peptides.

Immunization and Tests.—The immunization was carried out as described (8). 2 cc. of a solution containing 10 mg. in 1 cc. were used per injection.

On repeating precipitin tests with new GL and LG immune sera,[3] these sera appeared to be less specific than those described (1), and showed stronger cross-reactions between peptides containing the same terminal amino acid; *e.g.*, the GL serum with L_2 antigen and the LG serum with G_2 antigen. One of the LG sera gave a faint group reaction with the L_2 antigen (9), and a more marked precipitation was observed with a G_2 antigen and a GL serum. These results could not be explained entirely by the greater strength of some of the sera and may be due to the individually different response of the immunized animals, or possibly to the change in technique of preparing the immune sera.

Reactions with these and other immune sera on a series of "peptide-" and "amino acid-azoproteins" are summarized in Table I. In most cases distinct group reactions occurred whenever the antigens tested contained peptides with the same terminal amino acid as that present in the homologous azoprotein, and the reactions were, as a rule, the stronger the greater the similarity in structure of the terminal part of the peptide chain. On the other hand, in a number of combina-

[3] As in the previous paper the peptides will be designated by abbreviations such as G for glycine, G_2 for glycyl-glycine, GL for glycyl-leucine, etc.

TABLE I

To 0.2 cc. of the 1:500 diluted antigens (prepared with chicken serum) were added 2 capillary drops of immune serum. The dilution was made from a 5 per cent stock solution. The readings were taken after the tests had stood for 1 hour at room temperature.

Immune sera for	Test antigens prepared from chicken serum and the aminobenzoyl derivatives of																	
	G	L	G_2	GL	LG	L_2	G_3	G_2L	GLG	LG_2	G_4	G_2L	G_5	G_4L	G_2LG_2	LG_4	L_2G_3	Glutathione
G	++±	0	+	0	+	0	+	0	+±	+	+	0	±	0	±	±	0	tr.
L	0	++	0	++	0	+±	0	++	0	0	0	++	0	+±	0	0	0	0
GL No. 1	0	+	f.tr.	++	0	±	0	++	0	0	0	++	0	+±	0	0	0	0
GL No. 2	0	++	+	+++	0	+±	0	++±	0	0	0	++±	0	+++	0	0	0	0
LG No. 1	+	0	+±	0	++	0	+	0	++	+	±	0	±	0	±	±	tr.	+
LG No. 2	+	0	++	0	+++	f. tr.	+±	0	++	+	±	0	±	0	+	+	±	++
G_3 No. 1	±	0	++	0	0	0	+++	+	+±	++	++±	±	++	±	+±	++	±	f. tr.
G_3 No. 3	0	0	tr.	0	0	0	+±	0	0	tr.	+	0	+	0	±	+	0	0
LG_2 No. 1	tr.	0	++	0	±	0	++±	0	±	+++	++	0	+±	0	+++	++	+±	0
LG_2 No. 2	f. tr.	0	+	0	0	0	+	0	0	++	+	0	±	0	+	+	±	0
Glutathione	tr.	0	±	0	f. tr.	0	±	0	±	tr.	tr.	0	tr.	0	f. tr.	tr.	0	++

Tests with other dilutions of the antigens gave essentially similar results.

tions there were no or faint cross-reactions, although the substances tested have the same terminal amino acid (carrying the free carboxyl group) as the homologous antigens, *e.g.*: immune serum G + antigen L_3G_2; immune serum G_3 + antigens LG and glutathione; immune serum LG_2 + antigens G and glutathione; immune serum glutathione + antigens LG, G_2LG_2, L_3G_2.[4] Similarly, there was a marked difference in the reactions of the two tripeptides GLG and LG_2 when tested with LG_2 immune sera, and it is worth noting that the (pentapeptide) L_3G_2 antigen gave rather weak reactions with the immune sera LG and G_3, whereas it reacted distinctly with the immune serum for LG_2 to which it is more closely related chemically. Some overlapping reactions apparently were caused by the correspondence of amino acids not occupying the terminal position in the peptide chain; *e.g.*, one immune serum G_3 reacted moderately with the antigens G_2L, G_3L, G_4L.

In the experiments presented the only immune sera used were two sera for di- and three for tripeptides and the peptides used (apart from glutathione) were built up solely from glycine and leucine. These precipitin tests, still limited in extent, did not, on the whole, demonstrate great serological diversity. Significantly different were the results of inhibition tests carried out with nitrobenzoyl derivatives of the peptides. The readings are given in Table II. The data presented in Table II reveal a striking degree of specificity. Thus the sera GL and LG show marked group reactions only with the peptides which are very closely related in structure, namely the LG serum with the substance GLG and the GL serum with G_2L, G_3L and G_4L, but even these reactions are considerably weaker than the reactions with the homologous substances. Likewise, with the tripeptide sera G_3 (No. 1) and LG_2 marked cross-reactions occurred only when the structure of the substances tested was identical with that of the homologous substance with regard to the three amino acids at the free end of the chain. For instance, the sera G_3 distinguish between the substances LG_2 and G_3 or G_2LG_2 and G_5 and the serum LG_2 between GLG_2 and G_4, etc.

The greater specificity of the inhibition reactions, as compared to

[4] The glutathione immune serum did not react on an azoprotein made with glutaminic acid.

TABLE II

For the inhibition tests 0.2 cc. of the chicken serum antigens (diluted 1:500) was mixed with 0.05 cc. of a neutral solution of the nitrobenzoyl derivatives of the peptides (concentration 0.25 millimol in 10 cc.). To this 2 capillary drops of homologous immune serum were added. The control tube contained only antigen and immune serum. Readings taken after 5 minutes and 1 hour are presented in the table on the first and second line respectively.

Immune sera for	G	L	G_2	GL	LG	L_2	G_3	G_2L	GLG	LG_2	G_4	G_3L	GLG_2	G_5	G_4L	G_2LG_2	LG_4	C
G	0	+±	±	+	∓	+±	∓	+±	±	∓	∓	+±	+	+	+	∓	+	+±
	tr.	++	+±	++	++	++±	+	+±	+±	++	++	++	+±	++	++	+±	+±	++±
L	±	0	+	0	±	0	∓	0	∓	tr.	+	0	tr.	+	0	±	±	+
	+±	0	++	+	+±	∓	++	∓	+±	+	+±	+	+±	+±	∓	+±	+±	++
GL	++	+±	++	0	++	+	++	f. tr.	++	++	+±	f. tr.	+±	+±	tr.	+±	++	++
	+++	++±	++±	0	+++	++	+++	+	++±	++±	++±	+	++±	++±	+	++±	+++	+++
LG	+±	++	+±	++	0	+±	+±	++	±	+±	+±	++	+±	+±	++	++	+±	++
	++±	+++	++±	+++	0	++±	++±	+++	+±	++±	++±	+++	++±	++±	++±	++±	++±	+++
G_2	+±	++	+±	++	++	++	0	++	++	+±	tr.	+±	+±	±	++	+±	∓	++±
No. 1	++±	+++	++	+++	++±	+++	±	++±	++±	++	∓	++	++	+	++±	++	+	+++
G_3	+	+±	f. tr.	+	+±	+±	0	+±	+	tr.	0	+	±	f. tr.	+	±	f. tr.	+±
No. 2	+±	++	∓	+±	++	++	tr.	++	++	+	tr.	++	+	tr.	++	+	tr.	++
LG_2	+±	++	+±	++	+±	++	+	++	+±	0	+±	++	f. tr.	+±	+±	f. tr.	+±	++
No. 1	+++	+++	++±	+++	+++	+++	++	+++	+++	+	++±	+++	+±	+++	+++	+±	++	+++

that of the precipitin tests, cannot be explained on the basis of our present information and this question will require further study. It may be worth noting that with the precipitin tests, too, reactions of higher specificity may be obtained after absorption with heterologous antigens (9).

The investigations are being continued along the lines indicated in our previous communication(1).

SUMMARY

In continuation of previous studies immune sera for azoproteins made from aminobenzoyl dipeptides and tripeptides were tested with various peptide azoproteins by precipitin tests and with nitrobenzoyl derivatives of peptides by means of inhibition reactions. When examined with the latter method, the immune sera exhibited a high degree of specificity and permitted the recognition of distinctions among peptides of similar structure.

REFERENCES

1. Landsteiner, K., and van der Scheer, J., *J. Exp. Med.*, 1932, **55**, 781.
2. Fischer, E., *Ber. chem. Ges.*, 1904, **37**, 2486.
3. Abderhalden, E., and Zeisset, W., *Fermentforschung*, 1928–29, **10**, 544.
4. Abderhalden, E., and Schwab, E., *Fermentforschung*, 1928–29, **10**, 179.
5. Abderhalden, E., and Schweitzer, T., *Fermentforschung*, 1928–29, **10**, 341.
6. Abderhalden, E., and Fodor, A., *Ber. chem. Ges.*, 1916, **49**, 561.
7. Abderhalden, E., Dinerstein, L., and Genes, S., *Fermentforschung*, 1928–29, **10**, 532.
8. Landsteiner, K., and van der Scheer, J., *J. Exp. Med.*, 1934, **59**, 751.
9. Landsteiner, K., Die Spezifizität der serologischen Reaktionen, Berlin, Julius Springer, 1933. (Table 16, page 86; page 98.)