ON THE SEROLOGICAL SPECIFICITY OF PEPTIDES. III

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For the purpose of studying the serological behavior of synthetic substances of somewhat complex structure, which at the same time bear a relation to natural antigens, we have proceeded to investigate immune sera to azoproteins made from longer peptide chains than before (1, 2), that is, pentapeptides; the latter again were built up from glycine and *d,l*-leucine. Since in previous studies the terminal amino acid, with free carboxyl group, dominated to a considerable extent in the serological reactivity, peptide amides have now been included in order to eliminate the prominent influence of acid groups and to get information on the rôle played by other parts of the molecule in the reactions with immune bodies. The peptides used for the preparation of azo antigens were tetraglycyl-glycine, tetraglycyl-leucine, *d,l*-diglycyl-leucyl-glycyl-glycine and trileucyl-glycyl-glycine.[1]

EXPERIMENTAL

The preparation of a number of the substances used has been described previously (1, 2). With some of these compounds, namely the first four mentioned below, more convenient methods have now been utilized. Melting points were not corrected and mostly no attempt was made to recrystallize to constant melting point.

p-Nitrobenzoyl-Tetraglycyl-d,l-Leucine.—7.4 gm. of *p*-nitrobenzoyl-glycyl-glycine hydrazide (see below) were dissolved in 15 cc. 5/N HCl, 100 cc. of 50 per cent acetic acid, and 150 cc. of water, and were converted into the azide at 0–5°C. by slow addition of a solution of 2.6 gm. of sodium nitrite in 10 cc. of water. After ½ hour at 0–5°C. the azide was filtered off and washed with cold water until

[1] These compounds are hereafter designated respectively G_5, G_4L, G_2LG_2, L_3G_2, and the amides by the symbol Am. The preparation trileucyl-glycyl-glycine may well be a mixture of steric isomers although the nitrobenzoyl derivative was crystalline. (For other abbreviations see references 1, 2.)

free from acid. The azide was dissolved in 250 cc. of cold 85 per cent alcohol and the solution was added to a neutral solution of 7.4 gm. of diglycyl-*d*,*l*-leucine in 300 cc. of water. The mixture was stirred and 40 cc. of N/NaOH were added in small portions over a period of 1 hour to keep the solution slightly alkaline to litmus. After 2 more hours the solution was made weakly acid to litmus and concentrated *in vacuo* at 40° to a volume of 150 cc. If the solution became alkaline to litmus during the distillation, it was neutralized with HCl. After removal of a small amount of alkali insoluble material by filtration, the solution was made acid to Congo red. The sticky precipitate crystallized upon rubbing (needles). Yield 7.5 gm. It was recrystallized from water and again from 60 volumes of 50 per cent alcohol. Bushels of needles, m.p. unsharp with decomposition at 240°C. Analysis: Calculated for $C_{21}H_{28}O_9N_6$: N 16.54, found 16.49.

p-Aminobenzoyl-Tetraglycyl-d,l-Leucine.—The nitro compound was dissolved in 50 volumes of 75 per cent alcohol and reduced by means of palladium black and hydrogen at atmospheric pressure. The solution was evaporated to dryness *in vacuo* and the amino compound was recrystallized from 50 per cent alcohol. Microcrystalline. Yield 65 per cent. M.p. 165–166°C. Analysis: Calculated for $C_{21}H_{30}O_7N_6$: N 17.58, found 17.50.

p-Nitrobenzoyl-Diglycyl-d,l-Leucyl-Glycyl-Glycine.—This substance was prepared by the above method using 7.4 gm. of *p*-nitrobenzoyl-glycyl-glycine hydrazide and 7.4 gm. of *d,l*-leucyl-glycyl-glycine. After completion of the reaction and concentration *in vacuo* the solution was made acid to Congo red by addition of HCl and kept overnight in the ice box. The crystalline precipitate (needles) was filtered and recrystallized from 100 cc. of water. Yield 6 gm. M.p. 144–145°C. Analysis: Calculated for $C_{21}H_{28}O_9N_6$: N 16.54, found 16.61.

p-Aminobenzoyl-Diglycyl-d,l-Leucyl-Glycyl-Glycine.—The nitro compound was reduced by means of palladium and hydrogen as described, the alcoholic solution was evaporated to dryness *in vacuo*, the amino compound was dissolved in a small amount of methyl alcohol and precipitated by addition of dry ether. The precipitate became granular upon rubbing with more dry ether. Yield 90 per cent. Analysis: Calculated for $C_{21}H_{30}O_7N_6$: N 17.58, found 17.41.

Esters and Amides of Nitrobenzoyl Amino Acids and Nitrobenzoyl Peptides.—The nitrobenzoyl compounds (1,2) were converted into methyl esters by either dissolving or suspending the finely ground substance in 5 parts of absolute methyl alcohol and adding in portions an ether solution of diazomethane at 0–5°C., using a slight excess. The mixtures were allowed to stand at room temperature and after removal of the solvents the substances were further purified as described below. The esters were changed into amides by dissolving them in 100 parts (or more) of hot absolute methyl alcohol and saturating the solutions with dry ammonia gas at 0–5°C. The solutions were kept in a closed flask at room temperature for 48 hours and were then evaporated to dryness *in vacuo*; the last traces of ammonia were removed by again adding dry methyl alcohol and evaporating to dryness. The amides were further purified by recrystallization.

Reduction of Nitrobenzoyl Compounds.—The aminobenzoyl amino acid amides and aminobenzoyl peptide amides were prepared by suspending the finely ground nitro compounds in 50 volumes of 75 per cent ethyl alcohol (some were completely and others only partly dissolved) and reducing by means of palladium black and hydrogen at atmospheric pressure. After complete reduction no undissolved substance was left. The solutions were filtered and evaporated to dryness *in vacuo*. The amino compounds were further purified as described below.

p-Nitrobenzoyl-Glycine Ethyl Ester.—This was prepared by nitrobenzoylation of glycine ethyl ester by the method used previously for *p*-nitrobenzoyl-tyrosine ethyl ester (3). The chloroform solution of the nitrobenzoyl compound was evaporated to dryness *in vacuo* and the substance was freed from nitrobenzoic acid by dissolving in a small amount of hot chloroform and precipitating with 5 volumes of ether. It was recrystallized from 5 parts of absolute ethyl alcohol. Narrow platelets. Yield 16 gm. from 14 gm. of glycine ethyl ester hydrochloride. M.p. 141–143°C. Analysis: Calculated for $C_{11}H_{12}O_5N_2$: N 11.11, found 11.05.

p-Nitrobenzoyl-Glycine Amide.—Recrystallized from 30 parts of 80 per cent ethyl alcohol. Platelets. M.p. 239–240°C. Yield 80 per cent. Analysis: Calculated for $C_9H_9O_4N_3$: N 18.83, found 18.66.

p-Aminobenzoyl-Glycine Amide.—Recrystallized from 80 parts absolute methyl alcohol. Platelets. M.p. 225–226°C. Yield 70 per cent. Analysis: Calculated for $C_9H_{11}O_2N_3$: N 21.76, found 21.82.

p-Nitrobenzoyl-d,l-Leucine Methyl Ester.—Recrystallized from 20 parts of 50 per cent ethyl alcohol. Needles. M.p. 83–84°C. Yield 72 per cent. Analysis: Calculated for $C_{14}H_{18}O_5N_2$: N 9.52, found 9.48.

p-Nitrobenzoyl-d,l-Leucine Amide.—Recrystallized from 25 parts of 80 per cent ethyl alcohol. Platelets. M.p. 197–198°C. Yield 80 per cent. Analysis: Calculated for $C_{13}H_{17}O_4N_3$: N 15.05, found 14.86.

p-Aminobenzoyl-d,l-Leucine Amide.—Recrystallized from water. Platelets. M.p. 192–193°C. Yield 70 per cent. Analysis: Calculated for $C_{13}H_{19}O_2N_3$: N 16.87, found 17.05.

p-Nitrobenzoyl-Glycyl-Glycine Methyl Ester.—Recrystallized from 40 parts of 95 per cent ethyl alcohol. Platelets. M.p. 194–195°C. Yield 90 per cent. Analysis: Calculated for $C_{12}H_{13}O_6N_3$: N 14.24, found 14.36.

p-Nitrobenzoyl-Glycyl-Glycine Amide.—A solution of *p*-nitrobenzoyl-glycyl-glycine methyl ester in 150 parts of dry methyl alcohol was saturated with dry ammonia gas first at room temperature and subsequently at 0–5°C. From here on the general procedure was followed. Recrystallized from 120 parts of 80 per cent ethyl alcohol. Long needles. Upon rapid heating m.p. 257–258°C. with decomposition. Yield 80 per cent. Analysis: Calculated for $C_{11}H_{12}O_5N_4$: N 20.0, found 19.98.

p-Nitrobenzoyl-Glycyl-Glycine Hydrazide.—10 gm. of finely ground *p*-nitrobenzoyl-glycyl-glycine methyl ester were suspended in 100 cc. of absolute ethyl alcohol and 20 cc. of 100 per cent hydrazine hydrate were added. After shaking for 1 hour the mixture became very thick and the crystal form of the substance

had changed from the long platelets of the ester to hair-like needles of the hydrazide. The hydrazide was filtered off, washed with absolute alcohol, and recrystallized from 140 parts of 30 per cent ethyl alcohol. Needles. M.p. 250–251°C. Yield almost quantitative.

p-Aminobenzoyl-Glycyl-Glycine Amide.—Recrystallized from 80 parts of absolute methyl alcohol. Platelets. M.p. 211–212°C. Yield 70 per cent. Analysis: Calculated for $C_{11}H_{14}O_3N_4$: N 22.40, found 22.52.

p-Nitrobenzoyl-Glycyl-d,l-Leucine Methyl Ester.—Recrystallized from 50 per cent ethyl alcohol. Needles. M.p. 155–156°C. Yield 80 per cent. Analysis: Calculated for $C_{16}H_{21}O_6N_3$: N 11.97, found 12.20.

p-Nitrobenzoyl-Glycyl-d,l-Leucine Amide.—Recrystallized from 10 parts of absolute ethyl alcohol and also from ethyl acetate containing 10 per cent alcohol. Irregular platelets. M.p. 178–179°C. Analysis: Calculated for $C_{15}H_{20}O_5N_4$ N 16.67, found 16.70.

p-Aminobenzoyl-Glycyl-d,l-Leucine Amide.—Recrystallized from 20 parts of water. Needles. Yield 80 per cent. Analysis: Calculated for $C_{15}H_{22}O_3N_4$: N 18.30, found 18.18.

p-Nitrobenzoyl-Diglycyl-d,l-Leucine Methyl Ester.—Recrystallized from 20 parts of 50 per cent methyl alcohol. Needles. M.p. 177–178°C. Yield 70 per cent. Analysis: Calculated for $C_{18}H_{24}O_7N_4$: N 13.72, found 13.67.

p-Nitrobenzoyl-Diglycyl-d,l-Leucine Amide.—Recrystallized from 20 parts of 60 per cent ethyl alcohol. Platelets. M.p. 198–200°C. Yield 80 per cent. Analysis: Calculated for $C_{17}H_{23}O_6N_5$: N 17.81, found 17.95.

p-Aminobenzoyl-Diglycyl-d,l-Leucine Amide.—The substance was dissolved in a small amount of absolute methyl alcohol and precipitated from this solution by addition of 10 volumes of dry ether. Amorphous. Shrinks at 137°C. Yield 80 per cent. Analysis: Calculated for $C_{17}H_{25}O_4N_5$: N 19.28, found 19.38.

p-Nitrobenzoyl-d,l-Leucyl-Glycyl-Glycine Methyl Ester.—Recrystallized from 60 parts of 30 per cent ethyl alcohol. Platelets. M.p. 154–155°C. Yield 90 per cent. Analysis: Calculated for $C_{18}H_{24}O_7N_4$: N 13.72, found 13.55.

p-Nitrobenzoyl-d,l-Leucyl-Glycyl-Glycine Amide.—Recrystallized from 40 parts of 95 per cent ethyl alcohol. Needles. M.p. 201°C. Yield 75 per cent. Analysis: Calculated for $C_{17}H_{23}O_6N_5$: N 17.81, found 17.62.

p-Aminobenzoyl-d,l-Leucyl-Glycyl-Glycine Amide.—Recrystallized from 15 parts of absolute methyl alcohol. Platelets. M.p. 170–171°C. Yield 70 per cent. Analysis: Calculated for $C_{17}H_{25}O_4N_5$: N 19.28, found 19.18.

p-Nitrobenzoyl-Tetraglycyl-d,l-Leucine Methyl Ester.—Recrystallized from 30 parts of 50 per cent ethyl alcohol. Hair-like needles. M.p. 243–244°C. Yield 90 per cent. Analysis: Calculated for $C_{22}H_{30}O_9N_6$: N 16.09, found 16.05.

p-Nitrobenzoyl-Tetraglycyl-d,l-Leucine Amide.—*p*-Nitrobenzoyl-tetraglycyl-*d*, *l*-leucine methyl ester was dissolved in 300 parts of boiling dry methyl alcohol and the solution was saturated with dry ammonia gas first at room temperature and subsequently at 0–5°C. Then the general procedure was followed. Re-

crystallized from 30 parts of 50 per cent ethyl alcohol. Small needles. Yield 85 per cent. Analysis: Calculated for $C_{21}H_{29}O_8N_7$: N 19.35, found 19.24.

p-Aminobenzoyl-Tetraglycyl-d,l-Leucine Amide.—Recrystallized from 30 parts of absolute methyl alcohol. No distinct crystalline form. Shrinks at 180°C. and melts at about 185°C. Yield 85 per cent. Analysis: Calculated for $C_{21}H_{31}O_6N_7$: N 20.54, found 20.40.

p-Nitrobenzoyl-Diglycyl-d,l-Leucyl-Glycyl-Glycine Methyl Ester.—Recrystallized from water. Rosettes of needles. Yield 75 per cent. Analysis: Calculated for $C_{22}H_{30}O_9N_6$: N 16.09, found 15.98.

p-Nitrobenzoyl-Diglycyl-d,l-Leucyl-Glycyl-Glycine Amide.—Recrystallized from 20 parts of 50 per cent ethyl alcohol. Hair-like needles. M.p. 216–217°C. Yield 80 per cent. Analysis: Calculated for $C_{21}H_{29}O_8N_7$: N 19.35, found 19.18.

p-Aminobenzoyl-Diglycyl-d,l-Leucyl-Glycyl-Glycine Amide.—For purification the substance was dissolved in 15 parts of hot amyl alcohol. Amorphous substance which separated on cooling was filtered off and washed with dry ether. Yield 70 per cent. Analysis: Calculated for $C_{21}H_{31}O_6N_7$: N 20.54, found 20.38.

Azodyes.—The dyes used for inhibition tests were prepared by coupling the diazonium compounds, for ½ hour at 0–5°C., with an equimolar quantity of *m*-hydroxybenzoic acid in a solution kept alkaline by addition of a slight excess of sodium carbonate. After acidification and centrifugation the dyes were redissolved by means of dilute NaOH and after determination of the contents of dye stock solutions were made up to a concentration of 1:4 millimol of dye in 10 cc.

Immunization.—Rabbits were injected intravenously with 5 mg. of a suspension of horse azostromata (4) in 2 cc. After two to four courses of 6 daily injections each, with rest intervals of 1 week, the animals were bled on the 7th day following the last injection.

Tests.—Antigens used for the tests were made with chicken serum as described (2) using half as much of the diazonium compounds. The dilutions of the test antigens given in the tables are in terms of a 5 per cent stock solution. The tests were observed for 1 hour at room temperature and then kept overnight in the ice box. The intensity of the reactions is indicated as follows: 0, f. tr. (faint trace), tr. (trace), tr. (strong trace), ±, ±, +, +±, ++, etc.

In the inhibition tests, the appropriate amount of the solutions had to be determined by preliminary experiments.

Precipitin and Absorption Tests

The precipitin reactions of immune sera for the four pentapeptides are presented in Tables I and II; they are in keeping with those exhibited by sera to shorter peptide chains in that cross reactions occurred chiefly, but not exclusively (*e.g.* G_4L serum No. 2 on G_5 or on LG_4) with peptides having the same amino acid at the carboxyl

TABLE I

To 0.2 cc. of 1:500 diluted stock antigens (prepared with chicken serum) were added 2 capillary drops of immune serum. The readings given were made after the tubes had remained for 1 hour at room temperature.

Immune sera	Test antigens prepared from chicken serum and the aminobenzoyl derivatives of																			
	G	L	G_2	GL	LG	LL	G_3	G_2L	LG_2	GLG	G_4	G_3L	G_5	G_4L	G_2LG_2	L_3G_2	LG_4	Glut. G_2	Tyr. G_2	Glutathione
G_5	0	0	±	0	0	0	+±	0	tr.	0	+±	0	+±	0	+	0	+±	tr.	f. tr.	0
G_4L (No. 1)	0	±	0	+±	0	0	0	++	0	0	0	++	0	++	0	0	0	0	0	0
G_4L (No. 2)	0	+±	f. tr.	+++±	0	+±	+	+++	0	0	+	++++±	+	++++	f. tr.	0	⊥	0	±	tr.
G_2LG_2 (No. 1)	tr.	0	+	0	0	0	++	0	++	0	++	0	++	0	+++	+	+±	+	±	0
G_2LG_2 (No. 2)	0	0	⊥	0	0	0	+±	tr.	⊥	0	+	0	⊥	tr.	++	tr.	⊥	f. tr.	⊥	0
L_3G_2	0	0	+	0	0	0	+±	0	+±	0	+	0	+	0	+±	+±	+	⊥	f. tr.	0

TABLE II

To 0.2 cc. of the stock antigens, in a dilution of 1:500 unless specified, were added 2 drops of immune serum. 1 hour readings are tabulated.

Immune sera	G	L	G_2	GL	G_2L	LG_2	G_4L			G_2LG_2			G Am.	L Am.	G_2 Am.	GL Am.	G_2L Am.	LG_2 Am.	G_4L Am.			G_2LG_2 Am.		
							1:100	1:500	1:2500	1:100	1:500	1:2500							1:100	1:500	1:2500	1:100	1:500	1:2500
G_4L (No. 3)	0	tr.	0	+±	++	0	+±	++±	+		0		0	0	0	0	0	0	±	0	0		0	
G_2LG_2 (No. 2)	0	0	⊥	0	tr.	⊥		tr.		⊥	++	+	0	0	0	0	⊥	0		±		tr.	⊥	±
G_4L amide	0	0	0	0	0	0	0	tr.	tr.		0		0	±	0	+	++	0	++	++±	⊥	0	0	0
G_2LG_2 amide	0	0	0	0	±	0		f. tr.		tr.	+±	+	0	0	0	⊥	++	0	⊥	+±	f. tr.	+	++±	±

end. The cross reactions were definitely related to similarities in constitution. For instance, the L_3G_2 immune serum gave precipitation with G_2, not with LG or GLG and the reactions of G_4L immune serum No. 2 increased in strength in the sequence L, GL, G_2L, etc. Or, G_5 immune serum precipitated G_2 but not LG and, G_3 much more than LG_2.

The antisera for G_4L Am. distinguished sharply between G_4L Am. and G_2LG_2 Am. (Table II) although they differ only in the

TABLE III

To 0.04 cc. of the 1:100 diluted antigens were added 0.16 cc. absorbed immune serum (I.S.).

Two absorptions were carried out with G_5 azostromata, using 1.4 mg. with 1 cc. serum. With LG_2 the first absorption was made similarly, the second with half as much azostromata. The readings made after 15 minutes are shown in the first line, and those after 1 hour in the second line.

	G_3	LG_2	G_5	G_2LG_2
G_2LG_2 I.S. No. 1 absorbed with G_5	tr.	±	0	++
	tr.	+	0	++±
G_2LG_2 I.S. No. 1 absorbed with LG_2	±	0	+	++
	±	0	+±	+++

position of the leucine residue in the chain. Furthermore, the peptides and their corresponding amides in spite of great similarity in structure proved to be serologically different; G_4L and G_4L Am. showed weak, G_2LG_2 and G_2LG_2 Am. marked overlapping reactions. In addition cross precipitations were seen among amides as noted for peptides, however there was little overlapping between the two sorts of compounds. Of particular interest are observations that G_2LG_2 Am. sera react only faintly at best with LG_2 Am. antigen, which is identical as to the terminal part of G_2LG_2 Am., but do react (as to a lesser extent do G_2LG_2 sera) with G_2L Am., G_4L Am., and GL Am., which correspond to interior portions of the homologous substance. This is in contrast to the marked determinant influence of acid groups regularly encountered with free peptides, and the conclusion may be drawn that amide groups, though also strongly polar

(5), differ considerably from free acidic groups with regard to the effect on serological reactivity.

TABLE IV

The tests were put up as described in Table III. Two absorptions were made of G_2LG_2 immune serum, each time with 2.7 mg. G_5 azostromata and 1 cc. serum; for comparison, similar tests were made of the unabsorbed immune serum diluted with normal rabbit serum (see sub-table, readings after an hour). The L_3G_2 immune serum (1 cc.) was twice absorbed with G_3 (rabbit) azostromata, first with 0.7 mg. stromata, then with half as much. The reactions of the unabsorbed sera, although not directly comparable, are taken from Table I to indicate their relative strength.

The readings shown in the table were made after 1 hour and overnight in the ice box.

	G	G_2	G_3	G_4	G_5	LG_2	G_2LG_2	L_3G_2	LG_4	Glut. G_2	Tyr. G_2	Glutathione
G_2LG_2 I.S. No. 1 abs.	0	0	0	0	0	f. tr.	++	0	0	0	0	0
with G_5	0	0	0	0	0	tr.	++±	0	0	0	0	0
Unabsorbed	tr.	+	++	++	++	++	+++	+	+±	+	±	0
L_3G_2 I.S. abs. with G_3		0	0	0	0	±	+	+±	0	0	0	
		0	0	0	0	+±	+±	++±	0	0	0	
Unabsorbed		+	+±	+	+	+±	+±	+±	+	±	f. tr.	

	G_3	LG_2	G_5	G_2LG_2
G_2LG_2 I.S. No. 1 Diluted 1:2	++	+±	++	++±
Diluted 1:4	+	±	±	+±
Diluted 1:8	tr.	0	f. tr.	tr.

Previous experience with the complexity of immune sera (4) suggested absorption experiments which were carried out on a limited scale, using, as before, insoluble antigens (azostromata). With one serum each for G_5 and G_4L no clear-cut separation of antibody fractions was obtained by treatment respectively with azostromata G_3 and G_2L; but antibodies with different specificities actually were

demonstrable in G_2LG_2 sera on absorption with G_5 and LG_2 stromata (Table III); a similar result was observed when G_4L immune sera were absorbed with G_5 and G_4L Am.

The effect of partial absorption with heterologous peptide azostromata on the cross reactions of L_3G_2 and G_2LG_2 sera is seen from Table IV. With suitably absorbed serum L_3G_2 the supernatant fluid reacted only with the peptides containing a terminal LG_2 and with none of the other peptides. A still higher degree of specificity ap-

TABLE V

To 0.2 cc. of 1:500 dilutions of the antigens were added 3 capillary drops of immune serum or absorbed serum prepared by absorption for 2 hours at room temperature with 2 mg. of G_2LG_2 per cc. or (for control of non-specific absorption) G-azostromata. The readings given were made after 1 hour and overnight in the ice box.

Antigens	Immune serum for G_2LG_2 amide		
	Unabsorbed	Absorbed with G	Absorbed with G_2LG_2
G_2LG_2	++	++	0
	++±	++±	0
G_2LG_2 amide	++±	++±	0
	+++	+++	tr.

peared from the tests with immune sera G_2LG_2: absorption with G_5 in increasing amounts removed the cross reactions stepwise, and finally, using a proper amount of adsorbant, antibodies were left behind that were specifically adjusted to the entire structure (azobenzoyl) G_2LG_2 and did not act on the other related peptide antigens except for a slight reaction with LG_2. The control experiment, as tabulated, showed that the result is not due merely to diminution of the antibody content, as would be brought about by simple dilution.

As regards the overlapping reactions between peptides and corresponding amides (see Table II), the objection could be raised that the amides might have been hydrolyzed to some extent in the animal body with production of some free acid and antibodies thereto or, on the other hand, that such splitting occurred during the preparation of the azoantigens, resulting in a mixed antigen such as could give

rise to reactions with sera for the free peptides. However, these possibilities are ruled out by the following experiments. A serum for G_2LG_2 Am. was adsorbed with G_2LG_2 azostromata, and for control purposes with a non-reacting antigen as well, with the result that the antibodies which reacted with the amide were almost com-

TABLE VI

To 0.2 cc. of the given dilutions of stock antigen was added 1 capillary drop (in the case of G_4L amide antigen 3 drops) of immune serum either unabsorbed and diluted with normal rabbit serum or after being absorbed with G_4L Am. (2 mg. stromata per 1 cc. serum; 2 hours at room temperature). The readings given were made after 1 hour and after the tests had stood overnight in the ice box.

Immune serum G_4L (No. 2)	G_4L antigen						G_4L Am. antigen					
	1:50	1:100	1:200	1:400	1:800	1:1600	1:50	1:100	1:200	1:400	1:800	1:1600
After absorption with	0	±	+±	++±	++±	++	0	0	0	0	0	0
G_4L amide	0	±	++	++±	++±	++	f. tr.	f. tr.	f. tr.	0	0	0
Unabsorbed; diluted	tr.	±	+±	++	++	+±		+				
3:4 in normal rabbit serum	tr.	+	++	++±	++±	++		+±				
Unabsorbed; diluted	f. tr.	±	±	+±	+±	+		±				
1:2 in normal rabbit serum	tr.	±	+	++	++±	+±		+				

pletely exhausted by the peptide antigen (Table V). (With another serum only a small amount of antibodies was left behind.) This proves that the bulk and not only a minor portion of the antibodies contained in the amide serum reacts with the free peptide. Then in the case of G_4L immune serum (Table VI) the reactions with G_4L Am. antigen disappeared almost completely upon absorption with G_4L Am. stromata, while the homologous reaction was hardly diminished and this, taken in conjunction with the persistence of the reaction for G_4L Am. upon dilution of the unabsorbed G_4L immune serum, shows that in the G_4L Am. antigen there is no appreciable amount of G_4L and that the sera contain a small fraction of a special antibody cross reacting with the amide.

TABLE VII *a*

For the inhibition tests 0.05 cc. of neutral solutions of nitrobenzoyl peptides were mixed with 0.2 cc. of 1:500 dilutions of the stock chicken antigens, and homologous immune sera were then added (2 drops in the case of G_5 and 3 drops of G_4L and G_2LG_2); the concentrations of the inhibiting solutions (millimols in 10 cc.) were 1:8 for serum G_4L, 1:2 for serum G_2LG_2, and 1:4 for serum G_5. The control tube contained only antigen and immune serum.

Readings taken after 1 hour and after standing overnight.

	L	GL	G_2L	G_3L	G_5	G_4L	G_2LG_2	Control
G_4L I.S. (No. 4)	+	±	tr.	tr.	++	0	++	++
	++	+±	±	±	+++±	tr.	+++	+++±

	G_2	G_3	LG_2	G_4	G_5	LG_4	G_2LG_2	G_4L	Glut. G_2	Tyr. G_2	Control
G_5 I.S.	+	0	+	0	0	0	+	+	+	+±	++
	+±	±	+±	tr.	0	tr.	+±	+±	++	++	+++
G_2LG_2 I.S. (No. 3)	+	+	tr.	+	+	+	0	+±	+	+±	++
	++±	++±	++	++±	++±	++±	0	+++±	++±	+++	+++±

TABLE VII *b*

For the inhibition tests 0.05 cc. of solutions of peptide amide azodyes (concentration 1:33 millimol in 10 cc.) were mixed with 0.2 cc. of 1:500 dilutions of the stock G_4L Am. chicken antigen, and 2 capillary drops of immune serum were then added. Readings are given after 15 minutes and 1 hour, and after standing overnight.

	G Am.	L Am.	G_2 Am.	GL Am.	G_2L Am.	LG_2 Am.	G_4L Am.	G_2LG_2 Am.	G_4L	Control
G_4L amide I.S.	+±	+	+±	±	±	+±	0	+±	+	+±
	++	+±	++	±	±	++	tr.	++	+±	++
	++±	++±	++±	+±	+±	++±	tr.	++±	++±	++±

Inhibition Tests

Inhibition tests were carried out with nitrobenzoylated peptides;[2] peptide amides because of their poor solubility were converted into

[2] Positive inhibition reactions were also obtained with higher concentrations of non-acylated G_2LG_2. The compounds G_5, L_3G_2, G_4L, NO_2-benzoyl L_3G_2 could not be tested because of insufficient solubility.

easily soluble azodyes by diazotizing the aminobenzoyl derivatives and coupling to *m*-hydroxybenzoic acid. Results are presented in Tables VII *a* and VII *b*. When it is taken into account that only those substances are included which in the form of azoproteins gave positive precipitin reactions with the sera in question, these tests are

TABLE VIII

For the inhibition tests neutral solutions of nitrobenzoyl peptides were mixed with 0.2 cc. of 1:500 dilutions of the heterologous antigens before addition of the immune sera. In the case of G_4L serum, 0.05 cc. of solutions containing 1:8 millimol of the peptides in 10 cc. were tested, with G_2LG_2 serum, 0.1 cc. of solutions containing 1:12 millimol in 10 cc. 2 drops were used of G_4L and 3 drops of G_2LG_2 immune sera.

Readings taken after 1 hour and after standing overnight.

	L	GL	G_2L	G_5	G_4L	Control
G_4L I.S. No. 3 on G_2L antigen	+	±	tr.	+±	0	+±
	++	±	±	++±	tr.	++±

	G	G_2	G_2L	LG_2	G_5	G_2LG_2	LG_4	Control
G_2LG_2 I.S. No. 4 on G_2 antigen	+	±	+	±	±	0	±	+
	+±	+	+±	+	+	tr.	+	+±

rather strikingly specific for the homologous haptens. The distinction between the sera G_5 and G_2LG_2 differing in only one amino acid, may be mentioned and, again, the shading off of the reactions of sera G_4L, in the order G_4L, G_3L, G_2L, GL, and L (Table VII *a*). The amide azodyes did not inhibit the reactions of the peptide immune sera but showed sharply specific inhibitions with the homologous amide sera (Table VII *b*). A definite reaction was also seen with G_2LG_2 Am. serum and G_2LG_2 used either as nitrobenzoyl derivative or as azodye.

The inhibition method proved to be of advantage for characterizing the nature of antibodies operative in the cross precipitin reactions with heterologous antigens.

From such experiments in part presented for illustration (Table VIII), it appears that also those antibodies that produce precipitation with heterologous antigens are specifically related to the homologous substance in its entirety (page 713), that is to the one which served as determinant in the formation of the antibodies. When, therefore, one antigen is precipitated by several different immune sera, it can be shown by inhibition tests that the antibodies concerned are different, as in the instances given in Table IX.

TABLE IX

The procedure for the inhibition tests was that described in Table VII *a*; the concentrations of the inhibiting solutions were (as millimols in 10 cc.) 1:2, 1:8, and 1:16 as employed respectively with the three immune sera listed. 2 drops each were used of G_5 and G_2LG_2, and 3 drops of G_3 immune sera.

The readings presented were made after 15 minutes, 1 hour, and after standing overnight.

	G_3	G_5	G_2LG_2	Control
G_3 I.S. on G_3 antigen	0	tr.	±	++
	f. tr.	tr.	±	++±
	f. tr.	tr.	+±	++±
G_5 I.S. on G_3 antigen	tr.	0	+	+±
	±	tr.	+±	++
	+	±	++	++±
G_2LG_2 I.S. No. 2 on G_3 antigen	tr.	tr.	0	+
	±	±	0	+±
	±	±	0	+±

COMMENT

The four pentapeptides examined, although all contained only glycyl and leucyl residues, were distinct in their precipitin reactions when tested with various antigens; and replacement of even one of five glycines by leucine resulted in a noticeable alteration in serological properties. A pronounced serological change was brought about by the conversion of peptides into amides, and a similar modification was observed upon esterification of peptides (6).

The specificity of the sera was more conspicuous in inhibition than

in precipitin tests. The two kinds of reactions cannot readily be compared quantitatively, yet the apparently greater specificity of inhibition tests may be explained by the consideration that in inhibition reactions there is a competition between the weak affinity of the immune sera for heterologous haptens and the strong affinity to the homologous antigen, while in precipitin reactions there is present only one reacting substance and, moreover, precipitins of low activity can be aided and carried down in the precipitate by more potent antibodies (Heidelberger).[3]

From the absorption experiments described above one can conclude that the specificity of antibodies engendered by pentapeptides may be directed towards the molecule as a whole. In agreement with this, greater or at least equal inhibition of cross reactions was produced by the haptens homologous to the immune sera in comparison with those corresponding to the heterologous antigens tested (4). No evidence was found to demonstrate the formation of separate antibodies to several parts of the peptide structure, as had been observed with some other compounds in which highly determinant acid groups were linked to, and separated by a benzene ring (8). The difference in constitution between compounds of this sort and —CO—NH— chains may possibly be the reason for the disparity in the character of the antibodies formed. While thus it appears that antibodies may be formed which are specific for peptide chains consisting of five amino acid residues, it will be one of the next tasks to investigate, with the use of higher polypeptides, how large a structure in its entirety may be reflected in the configuration of antibodies, a question of considerable significance for the serological specificity of proteins.

The overlapping reactions of the peptide sera here reported were in general in accord with those formerly observed but a new fact emerged from the reactions of immune sera to a peptide amide (diglycyl-leucyl-diglycyl amide), namely strong cross reactions involving not the end groups but other parts of the molecule, a result likewise to be

[3] The lesser specificity reported for inhibition reactions with very simple compounds, *e.g.* substituted benzoic acids (7), may be ascribable to the circumstance that the "homologous" haptens did not so closely correspond to the antigens used in which the azodye structure probably plays a greater part in determining the reactivity than in the more complicated instances.

considered in protein reactions. These sera, unlike those for the corresponding free peptide, gave no or only weak overlapping reactions with peptide amides identical in their terminal groups with the homologous substance. It would seem, therefore, that the serological predominence of carboxyl groups (see also Goebel, 9) is suppressed by conversion into amides and that there is a definite difference in the serological significance of strongly polar amides and of dissociating acid groups. This may suggest that the combinations occurring in antigen-antibody reactions are not all of the same kind.

SUMMARY

Experiments are described dealing with immune sera to pentapeptides and peptide amides. Absorption and inhibition tests gave no indication of the presence in the immune sera of special antibodies for portions of a peptide molecule but the antibodies appeared to be specific for an entire pentapeptide even though the sera contained qualitatively different fractions. Marked disparity was found between the reactions of peptides and corresponding amides indicating differences between acid and other polar groups in their influence on serological specificity.

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