XXXI. PHENYL ISOCYANATE PROTEIN DERIVATIVES AND THEIR IMMUNOLOGICAL PROPERTIES.

III. THE AMINO-ACID DERIVATIVES AND SEROLOGICAL INHIBITION TESTS.

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IN previous papers [Hopkins and Wormall, 1933, 1, 2] the chemical and immunological properties of phenylcarbamido-proteins have been recorded. It is to be presumed that in these compounds the $C_6H_5.NH.CO$ -residue is attached to the free amino-groups of the lysine molecules. In view of the fact that serological inhibition tests have considerable value in the study of the constitution of chemically altered proteins, it was decided to make a more extensive study of the phenylcarbamido compounds. The phenyl- and *p*-bromophenylcarbamido-acids derived from many of the common amino-acids and similar compounds from α - and ϵ -amino-*n*-hexoic acid have therefore been prepared and have been used in precipitin and complement-fixation inhibition tests in the hope that more knowledge might be gained about the structure of proteins and the nature of the reaction between native proteins and phenyl isocyanates. In particular it was hoped that these tests would give more information about the free basic groups of the intact protein molecule.

Preparation of the phenylcarbamido-acids.

These compounds were prepared by the action of phenyl isocyanate (1.4 mol.) on the amino-acid (1 mol.) dissolved in water containing NaOH (1 mol.). Double quantities of NaOH were used for the preparation of the aspartic acid, cystine and tyrosine compounds and three times the normal quantity when lysine dihydrochloride was used. The amount of phenyl isocyanate was doubled for the reactions with lysine, cystine and tyrosine, since the first two contain two amino-groups per molecule and tyrosine has a hydroxyl group which under certain circumstances might react with the isocyanate.

The mixture of the isocyanate and the solution of the sodium salt of the amino-acid was well stirred at room temperature (15–18°) for 1 hour, 0.5 *N* NaOH being added where necessary to maintain a slight alkalinity. The solution was then filtered, and the phenylcarbamido-acid was precipitated from the filtrate by the addition of dilute HCl, with cooling where necessary. Most of these compounds were recrystallised twice from water or dilute alcohol and dried *in vacuo* over $CaCl_2$. It was not found possible to recrystallise the lysine compound, and it was purified by reprecipitation. The yield of the recrystallised product was usually quite satisfactory. The melting-points recorded are all uncorrected.

The phenylcarbamido-acids used were all known compounds except those obtained from α - and ϵ -amino-*n*-hexoic acids which had m.p. 140–141° and 126–127° respectively.

Preparation of the p-bromophenylcarbamido-acids.

These compounds were prepared by the action of *p*-bromophenyl isocyanate (1.4 mol.) dissolved in ether on the amino-acid (1 mol.) in slightly alkaline solution (1, 2 or 3 mol. of NaOH according to the amino-acid used, as in the case of the phenyl isocyanate compounds).

The reaction mixture was cooled in ice and shaken for about 1 hour, more NaOH being added as necessary to keep the solution alkaline. The solution was then filtered and the *p*-bromophenylcarbamido-acid precipitated as before and recrystallised twice. It was not found possible to recrystallise the lysine and cystine compounds. Bromine estimations (Carius) and melting-point determinations were carried out on all the products. The results are tabulated below.

Properties of the p-bromophenylcarbamido-acids.

Amino-acid	m.p. of product	% Br		Crystalline form
		Found	Calc.	
Glycine	Decomposes 200–210° without melting	29.2	29.3 (for $C_9H_9O_3N_2Br$)	Flat prisms
Alanine	181–183° (decomp.)	27.7	27.9 (for $C_{10}H_{11}O_3N_2Br$)	Flat prisms
Leucine	162–164° „	24.3	24.3 (for $C_{13}H_{17}O_3N_2Br$)	Prisms
α -Amino- <i>n</i> -hexoic acid	181–182° „	24.6	24.3 (for $C_{13}H_{17}O_3N_2Br$)	Prisms
ϵ -Amino- <i>n</i> -hexoic acid	170–172° „	24.0	24.3 (for $C_{13}H_{17}O_3N_2Br$)	Flat prisms
Lysine	176–177° „	28.5	29.5 (for $C_{20}H_{22}O_4N_4Br_2$)	Non-crystalline
Cystine	174–175° „	24.2	25.1 (for $C_{20}H_{20}O_6N_4S_2Br_2$)	Non-crystalline
Aspartic acid	175–176° „	24.5	24.2 (for $C_{11}H_{11}O_5N_2Br$)	Prismatic plates
Tyrosine	197–199° „	21.0	21.1 (for $C_{16}H_{15}O_4N_2Br$)	Flat prisms

The bromine values recorded in the above Table indicate that the normal reaction between *p*-bromophenyl isocyanate and the amino-group of the amino-acid occurs with all the amino-acids tested. In the cases of cystine and lysine, the calculations are based on the introduction of two of these groups into each molecule of the amino-acid. The Br values obtained with these preparations were significantly lower than the calculated values, and it appears probable that these preparations consisted mainly of the disubstituted compounds with smaller amounts of the monosubstituted compound or compounds.

Inhibition tests.

Landsteiner [1920] first showed that some simple substances could specifically inhibit the formation of a precipitate when certain antisera were added to corresponding antigens. In these tests a neutral solution of the substance, which is to be tested for inhibitory power, is mixed with the antigen solution. To this

mixture is then added an antiserum which normally gives a marked precipitate with the antigen. If no precipitate is produced (*i.e.* complete inhibition), and if the substance does not inhibit any other type of precipitin reaction (*e.g.* that between horse-serum and an antiserum against horse-serum), specific union between antibody and inhibiting substance has occurred. This substance might therefore be said to have groupings similar to the antigenically prominent groupings of the antigen. The value of such tests will be discussed later in this paper.

Precipitin inhibition tests. Precipitin tests were carried out under the conditions described previously [Hopkins and Wormal, 1933, 1, 2]. In the inhibition tests, 0.05 ml. of the antigen solution (0.025 % phenylcarbamido-horse-serum-proteins or more dilute solutions of phenylcarbamido-gelatin) was added to 0.20 ml. of a carefully neutralised solution of the inhibitory substance (0.01, 0.005 or 0.0025 *M*). To this mixture was added 0.10 ml. of the antiserum (antiserum against phenylcarbamido-horse-serum-globulin) and the degree of precipitin formation noted immediately and after 5 mins., $\frac{1}{2}$ hr. and 1 hr. These precipitin reactions were carried out at 37° except where the phenylcarbamido-gelatin compounds were used, these last-named compounds giving more pronounced precipitates at room temperature [Hopkins and Wormal, 1933, 2]. The results of the precipitin reactions are recorded as follows (in increasing degrees of precipitation): — (no reaction), f. tr. (faint trace), tr. (trace), $\pm$, +, $\pm\pm$, $\pm\pm\pm$, etc.

Complement fixation inhibition tests. To a series of tubes each containing 0.5 ml. of diluted antigen solution, the range comprising 1:20, 1:60, 1:180 down to 1:1,180,980 of a 5 % antigen solution, were added 0.10 ml. of a neutral 0.01 *M* solution of the inhibiting substance (in 0.9 % NaCl solution), 0.10 ml. of 1:5 guinea-pig-serum and 0.05 ml. of the antiserum. After these mixtures had been allowed to stand for $\frac{3}{4}$ to 1 hr. at room temperature, 0.5 ml. of a 4 % suspension of sensitised ox red cells (in 0.9 % NaCl) was added to each tube. The tubes were immersed in a water-bath at 37° and the extent of haemolysis noted at intervals. In many experiments similar series of tests were made with larger or smaller amounts of guinea-pig complement, the results being essentially the same as those with the amount recorded above.

Antigens and antisera.

The preparation of the antigens and antisera used for this investigation has been described in previous papers [Hopkins and Wormal, 1933, 1, 2]. The antigen concentrations (1:20, 1:100, etc.) refer to dilutions of a 5 % solution.

EXPERIMENTAL.

The phenyl- and *p*-bromophenyl-carbamido-acids were tested for the power to inhibit the reaction between phenylcarbamido-proteins and corresponding antisera. The antisera used were those against phenylcarbamido-horse-serum-proteins, whereas the antigen was some other phenylcarbamido-protein, either phenylcarbamido-chicken-serum-proteins or phenylcarbamido-gelatin. The last-named antigen proved of special value for this purpose, since with the antisera mentioned it gives marked precipitates in very high dilutions, and in these inhibition tests it is of advantage to reduce the amount of antigen to the minimum necessary to give a good precipitin reaction. In this way it is possible to reduce the amount of antigen competing with the inhibiting substance, and any

inhibitory action by the latter is usually more definite with the lower concentration of antigen.

These inhibition tests have been made with several concentrations of the antigen and antisera and with different amounts of the substances tested for inhibitory power. The last-named were usually used in 0.01 *M* solution in 0.9 % NaCl, this being about the maximum solubility for some of the substances. The results of a typical experiment are given in Table I, this Table giving readings with two concentrations of antigen and two concentrations of the inhibiting substances.

Table I. *Precipitin inhibition tests.*

Antigen: Phenylcarbamido-chicken-serum-proteins.

Immune-serum: Antiphenylcarbamido-horse-serum-globulin (No. 51).

Temperature: 37°.

Temperature: 31°.				
		Antigen solution		
	1 : 20	1 : 20	1 : 100	
	Solution of inhibiting substance			
	0.01 M		0.01 M	
Inhibiting substance	0.01 M	0.005 M		
NaCl	+ ±	+ ±	+ ±	+ ±
Glycine	+ ±	+ ±	+ ±	+ ±
Alanine	+ ±	+ ±	+ ±	+ ±
α-Amino- <i>n</i> -hexoic acid	+ ±	+ ±	+ ±	+ ±
Lysine	+ ±	+ ±	+ ±	+ ±
Phenylcarbamido-acetic acid	+	+	tr.	±
α-Phenylcarbamidopropionic acid	+	+	tr.	±
α-Phenylcarbamido- <i>n</i> -hexoic acid	+	+	tr.	±
αε-Diphenylcarbamido- <i>n</i> -hexoic acid	±	±	-	f.tr.
αε-Diphenylcarbamido- <i>n</i> -hexoic acid*	±	±	-	f.tr.
<i>p</i> -Bromophenylcarbamido-acetic acid	+ ±	+ ±	tr.	±
Readings after:	30 mins.	30 mins.	5 mins.	30 mins.

* Tests with one-half of the corresponding 0.01 or 0.005 *M* solutions.

From this table it will be seen that the phenylcarbamido-acids have a marked inhibitory influence on the precipitin reaction between phenylcarbamido-proteins and the corresponding antisera, and that the most pronounced effect is exhibited by the lysine derivative. Preliminary experiments of this nature were used in support of the view that the reaction between phenyl isocyanate and proteins involves the free amino-groups of the lysine molecules of the protein [Hopkins and Wormall, 1933, 1]. Since lysine has two NH₂-groups it seems possible that the corresponding phenylcarbamido-acid might be most efficient as an inhibitory agent because it contains two phenylcarbamido-groupings per molecule, but this explanation was soon excluded, for the lysine compound always gives much better inhibition than does twice the equivalent amount of the other α-phenylcarbamido-acids. In the experiments described in this paper, tests with one-half the equivalent amounts of the lysine compounds were always made, the results being identical with those obtained with the higher concentrations of these compounds.

The results given in Table I demonstrate the advantage of using as little antigen as possible for these inhibition tests, although it is necessary, of course, to obtain a good precipitate in the control tests, and they indicate also the added information which is gained by taking readings at various intervals of time. The results with *p*-bromophenylcarbamido-acetic acid show that this compound has some inhibitory action on the phenylcarbamido-precipitin reaction, but that the effect is less marked than that produced by phenylcarbamido-acetic acid.

One of the main objects of the work described in this paper was to obtain as much information as possible, using immunological methods, about the free basic groups of the protein molecule. For this purpose α - and ϵ -amino-*n*-hexoic acids and their phenyl- and *p*-bromophenyl-carbamido-derivatives were prepared and these compounds and the corresponding lysine compounds compared

Table II. *Precipitin inhibition tests.*

Immune serum: Antiphenylcarbamido-horse-serum-globulin (No. 51).

Temperature: 16°. (Readings after 30 mins.).

Inhibiting substance	Antigen		
	Phenylcarbamido-gelatin (1 : 500)		<i>p</i> -Bromophenyl-carbamido-gelatin (1 : 500)
NaCl	+ ±	+ ±	+
Alanine (lysine, or α - or ϵ -amino- <i>n</i> -hexoic acid)	+ ±	+ ±	+
Phenylcarbamido-acid corresponding to	{ alanine	tr.	tr.
	{ lysine	-	-
	{ α -amino- <i>n</i> -hexoic acid	tr.	tr.
	{ ϵ -amino- <i>n</i> -hexoic acid	-	-
Concentration of inhibiting substance:	0.01 <i>M</i>	0.0025 <i>M</i>	0.01 <i>M</i>

Note. None of the amino-acids used in this investigation, or the corresponding phenyl-carbamido-acids, had any inhibitory influence in these concentrations (0.01 *M* and more dilute solutions) on the precipitin reaction between horse-serum-globulin and an antiserum to horse-serum-globulin.

Table III. *Precipitin inhibition tests.*

Antigen: Phenylcarbamido-chicken-serum-proteins (1: 100) and phenylcarbamido-gelatin (1: 500).

Inhibiting substance: Neutralised 0.01 *M* solutions (in 0.9 % NaCl).

Antiserum: Antiphenylcarbamido-horse-serum-globulin (No. 94).

Temperature: 37° for the chicken-serum compound and 16° with phenylcarbamido-gelatin as antigen.

Inhibiting substance	Antigen			
	Phenylcarbamido-chicken-serum-proteins		Phenyl-carbamido-gelatin	
NaCl	++	++	+	+
Cystine (tyrosine, leucine, aspartic acid, α - and ϵ -amino- <i>n</i> -hexoic acids and lysine)	++	++	+	+
Phenylcarbamido-acid corresponding to	{ cystine	-	-	-
	{ tyrosine	tr.	tr.	-
	{ leucine	tr.	tr.	-
	{ aspartic acid	+	±	±
	{ α -amino- <i>n</i> -hexoic acid	-	-	-
	{ ϵ -amino- <i>n</i> -hexoic acid	-	-	-
	{ lysine	-	-	-
<i>p</i> -Bromophenyl-carbamido-acid corresponding to	{ lysine*	-	-	-
	{ cystine	tr.	±	f.tr.
	{ tyrosine	tr.	±	f.tr.
	{ leucine	±	±	-
	{ aspartic acid	±	±	f.tr.
	{ α -amino- <i>n</i> -hexoic acid	±	±	-
	{ ϵ -amino- <i>n</i> -hexoic acid	-	-	-
Readings after:	½ hr.	1 hr.	½ hr.	1 hr.

* *Cf.* Note to Table I.

Note. Smaller amounts of the phenylcarbamido-acids derived from cystine, tyrosine, leucine, aspartic acid and α -amino-*n*-hexoic acid gave slight inhibition only, whereas with the compounds from lysine and ϵ -amino-*n*-hexoic acid the inhibition was still complete.

as inhibitors of the phenylcarbamido-precipitin reaction. From the results of a typical experiment of this nature (*cf.* Table II), it appears that of these two monoamino-*n*-hexoic acids the ϵ -compound is the one which most resembles the lysine compound in its inhibitory power.

The results recorded in Table III are similar to those in Tables I and II. They show that all the phenylcarbamido-acids tested have a marked inhibitory effect on the precipitin reaction between phenylcarbamido-proteins and corresponding antisera, whereas the amino-acids themselves are inactive. The phenylcarbamido-acid derived from aspartic acid gave moderate inhibition only when compared with equivalent amounts of the corresponding compounds from glycine, cystine, *etc.*, but this is probably due to the fact that the preparation obtained by the action of phenyl isocyanate on aspartic acid is not very pure, and the recrystallised material represents a mixture of the free acid and the hydantoin [Paal and Zitelmann, 1903]. Thus all the phenylcarbamido-acids with the exception of the aspartic acid derivative gave complete or almost complete inhibition in the concentration used in the experiment recorded in Table III. With more dilute solutions of the inhibiting substances, the lysine and ϵ -amino-*n*-hexoic acid derivatives showed more marked inhibitory power than did those of the other amino-acids, as in the experiments recorded in Table II.

Table III also gives the results of tests with the *p*-bromophenylcarbamido-acids. These compounds, although not quite as effective as the corresponding phenyl compounds, possess a marked inhibitory capacity. The lysine derivative could not be used in these tests on account of its very slight solubility in water, but of the other compounds tested, the ϵ -amino-*n*-hexoic acid derivative was most effective. The introduction of bromine into the benzene ring of the phenylcarbamido-acids thus reduces somewhat but does not destroy the capacity of these substances to react with the antibodies to phenylcarbamido-proteins. The phenylcarbamido-acids are able similarly to inhibit the precipitin reaction between *p*-bromophenylcarbamido-gelatin (or the corresponding horse-serum compound) and antibodies to phenylcarbamido-proteins.

In Table IV a typical result is given showing the influence of some of the phenylcarbamido-acids on complement fixation tests, the other derivatives not

Table IV. *Complement fixation tests (inhibition tests).*

Antigen dilution	Inhibiting substance									
	Phenylcarbamido-acid corresponding to									
	NaCl	Glycine	α -Amino- <i>n</i> -hexoic acid	ϵ -Amino- <i>n</i> -hexoic acid	Lysine	Glycine	α -Amino- <i>n</i> -hexoic acid	ϵ -Amino- <i>n</i> -hexoic acid	Lysine	
1:20	4	4	4	4	4	4	4	4	4	4
1:60	4	4	4	4	4	4	4	4	4	4
1:180	3	4	4	4	4	4	4	4	4	4
1:540	—	2	2	4	3	4	4	4	4	4
1:1620	—	—	—	—	—	1	—	—	—	—
1:4,860	—	—	—	—	—	—	—	—	—	—
1:14,580	—	—	—	—	—	—	—	—	—	—
1:43,740	—	—	—	—	—	—	—	—	—	—
1:131,220	—	1	—	—	—	—	—	—	—	—
1:393,660	2	4	—	—	—	—	—	—	—	—
1:1,180,980	4	4	—	1	—	1	—	1	—	1
Control (NaCl)	4	4	4	4	4	4	4	4	4	4
Reading after (hr.)	$\frac{1}{4}$	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{2}$

In the above table, 4 represents complete haemolysis, — no haemolysis, and 3, 2 and 1 intermediate degrees of haemolysis. Phenylcarbamido-gelatin was the antigen used for these tests and the antiserum was one against phenylcarbamido-horse-serum-globulin.

quoted in this Table giving results similar to those with that prepared from glycine. Thus, these compounds are capable of effecting marked inhibition of the fixation of complement by mixtures of phenylcarbamido-gelatin and antisera to phenylcarbamido-proteins. This other type of inhibition test, involving inhibition of complement fixation, appears to give even more conclusive results than do the corresponding precipitin inhibition tests, and they confirm the finding that the phenylcarbamido-acids from lysine and ϵ -amino-*n*-hexoic acid are the most active of the amino-acid derivatives tested. In these complement fixation tests, as with the corresponding precipitin inhibition tests, experiments were made repeatedly to show that the inhibitory action of the phenylcarbamido-acids is specific, and that these compounds do not inhibit the interaction (in complement fixation and precipitin tests) between other antigens and their antibodies.

DISCUSSION.

Specific inhibition of the precipitin reaction by simple substances was first described by Landsteiner [1920], and since then this reaction has been used with much success by Landsteiner and van der Scheer [1928; 1931; 1932, 1, 2] and by other authors. Specific inhibition is obtained when the added substance contains a grouping identical with, or related to, that which is antigenically active in the antigen. Thus, *p*-amino- and *p*-bromo-benzoic acids inhibit the precipitin reaction between *p*-aminobenzoic acid-azo-protein and its antibody, but much less inhibition is exerted by *o*-amino- or *o*-bromo-benzoic acid [Landsteiner, 1920]. In a similar manner, the reaction between azo-proteins formed by the coupling of a protein with diazotised aminobenzoyl-dipeptides (e.g. *p*-aminobenzoylglycylglycine) and antibodies to these compound proteins is specifically inhibited by the corresponding *p*-amino- or *p*-nitro-benzoyl-dipeptide [Landsteiner and van der Scheer, 1932, 1]. In many instances therefore it has been shown that simple substances can unite with antibodies and prevent, partially or completely, the reaction with the homologous antigen, and such tests are very helpful in studies on the interaction between antigen and antibody and in investigations concerned with immunological specificity. Such tests may also be used for more strictly chemical purposes. Thus, the inhibition of the reaction between iodinated proteins and their antisera by 3:5-dihalo-genated tyrosine furnished very strong evidence in support of the view that the method of iodination used resulted mainly in the introduction of two iodine atoms into the 3:5-positions of each tyrosine grouping [Wormall, 1930].

From the investigations referred to above it will be seen that these inhibition tests can reveal much about the nature of groupings in chemically altered proteins, or proteins to which new groupings have been attached, and it appears highly probable that they will help considerably in attempts to learn more about the intact protein molecule. The tests do not involve any drastic treatment of the material to be tested, and they also allow the recognition of substances which cannot readily be isolated in the pure condition. Thus "inert" substances do not interfere with the inhibitory action of other substances, e.g. the specific inhibition of the phenylcarbamido-precipitin reaction by phenylcarbamido-acids, described in this paper, is not affected by the presence of unchanged amino-acids, *etc.* The tests can readily be used, therefore, to detect the presence of certain substances in a fairly complex mixture. They can also be used, in addition to the ordinary precipitin tests, in studies on spatial configuration [Landsteiner and van der Scheer, 1932, 3].

The work described in this paper has been concerned mainly with the free amino-groups of the protein molecule. Phenyl isocyanate readily reacts with

peptones as was shown by Raper [1907], who first described these compounds, and a similar reaction occurs with native proteins [Hopkins and Wormall, 1933, 1, 2]. The reaction with native proteins will obviously be more restricted, since the number of free amino-groups in the molecule of a protein of this type is usually rather small, and with a protein like zein, which is lacking in these basic groups, no reaction occurs. In the phenylcarbamido-proteins therefore the new groups appear to be attached to the free amino-groups only, and evidence of a chemical and immunological nature was put forward in support of the view that the main new grouping in these protein compounds is attached to the ϵ -amino-group of lysine [Hopkins and Wormall, 1933, 1]. Lysine however is a diamino-acid (α -diamino-*n*-hexoic acid), and it would be very helpful if we could determine whether the new groupings are attached to the α - or the ϵ - or both the α - and ϵ - amino-groups of the lysine molecules: in other words, which amino-groups are free in the intact protein. The chemical evidence available suggests that the free grouping is the ϵ -amino-group [cf. Van Slyke and Birchard, 1913; Edlbacher, 1921; Goldschmidt and Kinsky, 1929; Kapfhammer, 1930; Abderhalden and Schweitzer, 1931].

This view about the free amino-groups of the intact protein molecule receives very strong support from the immunological evidence presented in this paper. Inhibition tests (precipitin and complement fixation) show that α -diphenylcarbamido-*n*-hexoic acid combines very readily with antibodies to phenylcarbamido-proteins, and that similar phenylcarbamido-acids derived from the common amino-acids (glycine, cystine, tyrosine, *etc.*), although they are quite active in this respect, do not show by any means the same degree of activity as the lysine derivative. Furthermore, a comparison of the phenylcarbamido-acids from α - and ϵ -amino-*n*-hexoic acids has demonstrated that the latter behaves like the lysine derivative and the former like those of the other amino-acids. Thus the configuration of ϵ -phenylcarbamido-*n*-hexoic acid shows most resemblance to that configuration in phenylcarbamido-proteins which reacts with phenylcarbamido-antibodies, which leads one to the conclusion that a large number of the ϵ -amino-groups of the lysine components of the protein are free. The marked difference between the inhibitory powers of the lysine and the ϵ -amino-*n*-hexoic acid derivatives on the one hand, and derivatives of the other amino-acids on the other, suggests that the majority, if not all, of the ϵ -amino-groups of the lysine are free, but this conclusion must await further experimental evidence. Although this particular problem might not be capable of final solution by serological tests similar to those used in this investigation, sufficient has been said here to indicate the value of such tests in studies on the chemistry of proteins.

SUMMARY.

1. Phenyl and *p*-bromophenyl isocyanate derivatives of several amino-acids have been prepared, including those of α - and ϵ -amino-*n*-hexoic acids.

2. Inhibition by these phenylcarbamido-acids of the precipitin reaction between phenylcarbamido-proteins and the corresponding antisera has been studied. Similar inhibition of the complement fixation reactions has been obtained.

3. The phenylcarbamido-acids from lysine and ϵ -amino-*n*-hexoic acid give complete inhibition, whereas equivalent amounts of the derivatives of α -amino-*n*-hexoic acid, leucine, alanine, tyrosine, *etc.*, give moderate inhibition only.

4. These serological inhibition tests offer, therefore, strong support for the view that the free amino-groups of the intact protein molecule are the ϵ -amino-groups of the lysine molecules.

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