XCVII. PHENYL *ISO*CYANATE PROTEIN COMPOUNDS AND THEIR IMMUNOLOGICAL PROPERTIES.

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The chemistry of antigens can be studied in several different ways, and each method has its own definite advantages. On one hand, the protein molecule can be altered by the action of enzymes, acids or alkalis, or chemical alteration may be effected by nitration, iodination, oxidation, *etc.*, and a study of protein specificity made. Another method [Landsteiner and Lampl, 1917; 1918; Landsteiner, 1920; Landsteiner and van der Scheer, 1927; 1929] has involved the attachment to proteins of groupings of well-defined chemical nature by the interaction of various diazo-compounds with the protein to give azo-proteins, and this second method has been utilised for a more general study of the relationship between antigenic specificity and chemical structure. Full discussions of the results of the application of these and other methods can be found elsewhere [Hartley, 1931; Landsteiner, 1927; 1933; Wells, 1929].

The experiments with the azo-proteins have yielded results of great value and have very materially increased our knowledge about the specificity of antigens. It would appear desirable, therefore, that other methods for introducing into the protein molecule new groupings of definite structure should be utilised, but suitable methods of this nature are not readily found. Any treatment which is at all drastic and which involves the use of strong acids, alkalis or oxidising agents may be objected to on the grounds that hydrolytic and other changes necessarily accompany the introduction of new groups. The ideal method would be one which resulted in the introduction of a determinable number of new groups into a definite part of the protein molecule unaccompanied by any other change (hydrolytic, oxidative, *etc.*). With this object in view we have investigated the reaction between proteins and phenyl *iso*cyanates. Raper [1907] prepared phenyl *iso*cyanate derivatives of various fractions of the peptones from serum-albumin and obtained well-defined products with characteristic melting-points. The conclusion was reached by Raper that these compounds are definite chemical individuals. More recently, Folley [1932] has prepared the phenyl *iso*cyanate derivative of plastein. There appeared to be no definite evidence, however, that native proteins could react with phenyl *iso*cyanate in this way, although it seemed most probable that a reaction of this type would take place. Preliminary experiments were therefore carried out to determine whether phenyl *iso*cyanate would react with proteins such as serum-globulin and caseinogen. Since the main object of this investigation was concerned with the immunological study of these protein compounds, certain slight modifications had to be introduced into the method mainly with the idea of maintaining during the reaction a p_H between 7 and 8. The experiments were successful in yielding new protein derivatives which were strongly antigenic and which gave interesting results from the immunological view-point.

In the description below, this investigation is divided into two parts, the first part dealing with the preparation of these phenylureido-compounds and the study of their chemical properties, and the second part with the immunological experiments.

I. The preparation and properties of the phenylureido-proteins.

Preparation of phenylureido-caseinogen.

"Light white caseinogen" (B.D.H.) was purified by precipitation from an alkaline solution by the addition of acetic acid. 30 g. of caseinogen were made into a smooth paste with about 500 cc. of distilled water, dilute NaOH added to bring the reaction between p_H 9 and 10 and the solution centrifuged. The caseinogen was precipitated from the supernatant solution by means of dilute acetic acid and filtered off. The precipitate was dissolved in about 500 cc. of distilled water with the aid of dilute NaOH, the p_H being kept below 10, and, after the solution had been centrifuged to remove the undissolved matter, the caseinogen was precipitated again with acetic acid. The final product was taken up in about 500 cc. of distilled water, dilute NaOH added to give p_H 9 and the solution centrifuged.

To 50 cc. of this purified caseinogen solution were added 100 cc. of a phosphate buffer of p_H 8·0 (250 cc. of 0·2 *M* KH_2PO_4 *plus* 234 cc. of 0·2 *N* NaOH, made up to 1 litre). The mixture was then cooled in ice and 2 cc. of phenyl *iso*cyanate added slowly with continuous stirring. The reaction of the fluid was determined at frequent intervals by means of indicators, and 0·2 *N* NaOH was added when necessary to restore the p_H to 8·0 approximately. After having been stirred for about 1 hour, the mixture was adjusted to p_H 8·0 again and centrifuged, the precipitate being discarded. 2 % acetic acid was then added to the solution to give maximum formation of a flocculent precipitate. This product was removed by centrifuging and redissolved in about 200 cc. of distilled water with the addition of sufficient 0·2 *N* NaOH to give a clear solution. When this and similar precipitates were dissolved in water with the aid of dilute NaOH, the alkali was added very cautiously and frequent tests were made to ensure that the reaction of the solution did not become more alkaline than about p_H 8·5. This precaution was taken in order that there might not be any significant loss of antigenic power due to the action of alkali. At p_H 11, the relatively labile serum-proteins appear to suffer little loss of antigenic power at about room temperatures over a period of several hours [Johnson and Wormall, 1932], and since the solutions referred to in this paper were rarely allowed, even for a short period, to become more alkaline than p_H 8·5 and never more alkaline than p_H 9·5, it might justifiably be concluded that no loss of antigenic property or change in specificity occurred as a result of the action of alkali.

The phenylureido-caseinogen was reprecipitated by the addition of dilute acetic acid, the precipitate collected by centrifuging and redissolved as before. A third precipitation was made, and the precipitate finally dissolved in 30 to 40 cc. of distilled water with the addition of just sufficient 0·2 *N* NaOH to give a clear solution at about p_H 7·6.

Preparation of phenylureido-gelatin.

1 g. of gelatin (Coignet's "Gold Label") was dissolved in a warmed mixture of 65 cc. of distilled water and 65 cc. of the phosphate buffer of p_H 8·0. The solution was then cooled in ice, 0·5 cc. of phenyl *iso*cyanate was added slowly

and the whole stirred for about one hour, the reaction of the mixture being maintained at about p_H 8·0 by the addition of 0·2 *N* NaOH as above. At the end of this period the insoluble matter was discarded and the phenylureido-gelatin in the supernatant solution obtained as a gelatinous precipitate by the addition of acetic acid. Purification of this product was effected as for the corresponding caseinogen compound by two further precipitations with acetic acid. In the preparation of this gelatin compound and in that of *p*-bromophenyl-ureido-gelatin, the precipitates dissolved in water at p_H 7·5 to 8·0 with some difficulty in the cold, but solution was readily effected at 30 to 35°. The solutions were not allowed to remain at these higher temperatures longer than was absolutely necessary.

Preparation of phenylureido-horse serum-globulin.

The serum-globulins were separated as follows. 100 cc. of horse serum were diluted with about 900 cc. of distilled water, the solution cooled in ice and CO_2 passed in to give maximum precipitation. This suspension was then allowed to stand in the ice-chest for 24 to 48 hours, the supernatant fluid syphoned off, and the globulin precipitate separated by centrifuging the remainder of the fluid. This precipitate was then dissolved in about 100 cc. of 0·9 % NaCl solution, 0·2 *N* NaOH being added to adjust the p_H to about 7·5, excess of alkali being carefully avoided. From this solution the globulin was again precipitated by CO_2, the suspension allowed to stand for 24 to 48 hours in the ice-chest and the centrifuged deposit dissolved in about 40 cc. of 0·9 % NaCl solution, the reaction of the final solution being adjusted to about p_H 7·5.

To 100 cc. of this solution of horse serum-globulin were added 100 cc. of phosphate buffer of p_H 8 and 10 cc. of 10 % NaCl solution (added to maintain the concentration of NaCl at approximately 0·9 %). The mixture was cooled in ice and 3·5 cc. of phenyl *iso*cyanate added slowly to the well-stirred solution. The reaction of the mixture was maintained as before at about p_H 8·0, and after one hour 200 cc. of 0·9 % NaCl were added, and the fluid was centrifuged. The precipitate was washed twice with about 100 cc. of 0·9 % NaCl solution with 0·2 *N* NaOH added to give p_H 8, and the washings were added to the original supernatant solution. (This washing of the precipitate was only carried out when it was found desirable to improve the yield of phenylureido-protein.) The phenylureido-globulin was then precipitated by acetic acid and purified by two further precipitations with acetic acid as described for phenylureido-caseinogen, the precipitate being dissolved in 0·9 % NaCl solution each time instead of in distilled water: the solution of the globulin preparation was never allowed to become more alkaline than p_H 8·5. The final product was dissolved in about 70 cc. of 0·9 % NaCl solution with sufficient 0·2 *N* NaOH to give p_H 7·5.

Preparation of p-*bromophenylureido-protein compounds.*

Similar *p*-bromophenylureido-compounds of caseinogen, gelatin and horse serum-globulin were made by the action of *p*-bromophenyl *iso*cyanate in ethereal solution on a solution of the protein in a phosphate buffer at p_H 8.

100 cc. of caseinogen solution, containing approximately 3·5 g. of twice reprecipitated caseinogen (see above) were mixed with 200 cc. of phosphate buffer of p_H 8·0, and the mixture was cooled in ice. A filtered solution of 2·5 g. of *p*-bromophenyl *iso*cyanate in about 30 cc. of ether was then added and the cooled mixture stirred vigorously for about 1½ hours. The p_H was maintained at about 8·0, and *p*-bromophenylureido-caseinogen was precipitated and purified

by two further precipitations as described for the phenylureido-compounds. Similar *p*-bromophenylureido-compounds of gelatin and horse serum-globulin were made.

In order that determinations of the bromine content might be made, it was necessary in some cases to have dried and purified preparations of these *p*-bromophenylureido-compounds. For this purpose a portion of the solution of the thrice precipitated preparation was treated with three times its volume of absolute alcohol and sufficient dilute acetic acid added to give maximum precipitation. The precipitate was then washed with absolute alcohol, an alcohol-ether mixture and finally with ether. This preparation was then dried *in vacuo* over $CaCl_2$, powdered and extracted with ether in a Soxhlet apparatus for 100 hours. The material was powdered again and extracted with ether for a further 100 hours. The final product was dried *in vacuo*, and bromine estimations were made by the Carius method, the following values being obtained:

p-bromophenylureido-caseinogen:	4·1 % Br (1st preparation)
	5·0 % Br (2nd preparation)
p-bromophenylureido-gelatin:	2·6 % Br (1st preparation)
	2·2 % Br (2nd preparation)

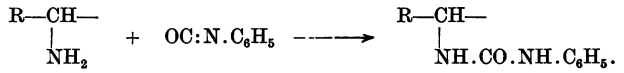
Nitrogen determinations. Total nitrogen determinations were made by the micro-Kjeldahl method. The amount of *amino-nitrogen* present in the various solutions was determined (*a*) by the method of Van Slyke (micro-apparatus), and (*b*) by Sørensen's formaldehyde titration. For the last-named determination, the solution was adjusted to p_H 7 (neutral red range), neutralised formaldehyde solution added, and the amount of 0·02 or 0·1 *N* NaOH required to bring the solution to p_H 9 (phenolphthalein range) was then measured.

Discussion.

By the method described above, a reaction has been induced between phenyl *iso*cyanate and native proteins, similar to that between certain peptone fractions and phenyl *iso*cyanate described by Raper [1907]. For the purposes of the investigation described here it was necessary to prevent gross changes in the p_H of the solution, *i.e.* to avoid, if possible, the use of strong alkali and to ensure that the acid produced during the reaction did not cause any marked acidity. After preliminary investigations it was found that a phosphate buffer of p_H 8·0 was admirably suitable for this purpose, especially if 0·2 *N* NaOH was added at intervals during the reaction in order to maintain the p_H at about 8·0. With these modifications the reaction appears to be ideal for immunological purposes, since a chemical change in the protein molecule is effected rapidly in approximately neutral solutions and at relatively low temperatures (4 to 8°). After being cooled and stirred for about one hour, the mixture of phenyl *iso*cyanate, protein and phosphate buffer contained some solid diphenylurea which could be largely removed by centrifuging, leaving a solution which contained a protein precipitable by dilute acetic acid at about p_H 4 to 4·5. This conversion of proteins such as the serum-proteins and gelatin which are not normally precipitated to any significant extent on the addition of dilute acetic acid (provided sodium chloride or some similar salt is present to prevent precipitation of the serum-globulin) into compounds which are readily precipitated by dilute acids suggests a chemical alteration in the protein molecule. Changes such as denaturation might possibly account, however, for this difference in solubility and it was considered desirable that further information about

the action of phenyl *iso*cyanate on proteins should be obtained. Phenyl *iso*cyanate readily reacts with amino-acids in alkaline solution and will also react with peptones, as shown by Raper [1907].

The reaction between phenyl *iso*cyanate and proteins would appear to be concerned mainly with the free amino-groups of the protein molecule to give a reaction of the following type:



Such a reaction would result in a diminution in the amount of free amino-nitrogen, and, assuming that no other change occurs, this diminution would be a measure of the extent of the reaction and the number of new groupings introduced. The results of some of the determinations of free amino-N, calculated as the percentage of total N present as free amino-N, are given in Table I, and from these figures it will be seen that there is a very considerable loss of free amino-N when these proteins are acted upon by phenyl and *p*-bromophenyl *iso*cyanates. Indeed, with caseinogen about 80 to 90 % of the free amino-N disappears when the protein has reacted with these *iso*cyanates.

The figures given in Table I do not show in every instance a very marked agreement between the results obtained with the two different methods for amino-N determination, but this disagreement is in accord with the observations of Wilson [1923] who compared these two methods using solutions of native and derived proteins. Wilson reached the conclusion that the method of Sørensen seems to be less susceptible to error than is Van Slyke's method in the analysis of proteins. From Table I it will be seen that the values given by Van Slyke's method were usually lower than those by the formaldehyde method, and these lower results with the former method are probably mainly due to precipitation of protein by the acid used in the determination.

Table I. *Amount of free amino-N in some proteins and their phenylureido- and* p*-bromophenylureido-derivatives.*

Recorded as the % of total N present as free amino-N.

Protein	Preparation	Method of amino-N determination	Unchanged protein %	Phenyl-ureido-protein %	*p*-Bromo-phenyl-ureido-protein %
Horse serum-globulin	1	Van Slyke	3·8	1·3	—
	2	Van Slyke	4·9	2·4	0·2
Caseinogen	1	Van Slyke	5·3	0·4	0·6
		Sørensen	5·5	0·6	0·7
	2	Van Slyke	4·4	0·6	0·1
		Sørensen	6·1	1·3	0·6
Gelatin	1	Van Slyke	2·3	1·7	0·5
		Sørensen	2·85	0·8	0·6
	2	Sørensen	3·0	0·8	1·2

This invariable diminution in free amino-N would appear to show that a reaction occurs between the phenyl *iso*cyanate and the free amino-groups of the protein molecule, but this evidence would not by itself be completely satisfactory, nor do the results exclude the possibility of additional types of reaction. The proof that one type of reaction only occurs, involving the formation of phenylureido-groupings, will be more complete if it can be shown that the number of new groups entering the protein molecule is equal to the number of

free amino-groups which have disappeared. Attempts to correlate these two have been made with the *p*-bromophenyl *iso*cyanate protein compounds, with fairly satisfactory results. From the diminution in free amino-N (Sørensen's method) when caseinogen is converted into the *p*-bromophenylureido-compound, and using the value 15·62 % for the N content of caseinogen [Plimmer, 1917, p. 131], it was calculated that one sample of the dried *p*-bromophenylureido-caseinogen obtained should have 3·9 % of bromine; the observed value was 4·1 %. A second sample of this compound gave "calculated" and observed values of 4·4 and 5·0 % respectively. With the gelatin preparations, samples of dried *p*-bromophenylureido-gelatin which should have contained 1·8 and 2·4 % of bromine (from the diminution in amino-N by the formaldehyde method and taking the figure 18·00 % for the N content of gelatin [Plimmer, p. 131]) gave values of 2·2 and 2·6 % respectively. This agreement may be considered quite satisfactory, and thus these results appear to show that the only reaction concerned is that between the *p*-bromophenyl *iso*cyanate and the free amino-groups. The observed values are somewhat higher than the values calculated from the amino-N diminution, but the divergence is not sufficient to indicate the introduction of bromine-containing groups into other parts of the protein molecule. The small differences might easily be due to the presence of other bromine-containing substances (di-*p*-bromophenylurea, *etc.*) in the dried *p*-bromophenylureido-proteins, in spite of the fact that continuous extraction with ether, with occasional repowdering of the material, was carried out for over 200 hours.

The chemical evidence appears, therefore, to show that a reaction occurs between phenyl and *p*-bromophenyl *iso*cyanates and the free amino-groups of the protein molecule to form phenylureido-protein compounds, and it is difficult from the purely chemical aspect to obtain more definite information about this reaction. It seems probable that the most satisfactory evidence will be that obtained as a result of the application of immunological methods. The immunological results will be discussed more fully in the second part of this paper, but it might be mentioned here that they indicate that a chemical alteration in the protein molecule takes place as a result of the action of phenyl and *p*-bromophenyl *iso*cyanates and that the reaction involves the free amino-groups of the lysine molecules of the protein.

From the chemical point of view, further studies are being made of the phenylureido- and *p*-bromophenylureido-protein compounds. They are readily soluble in water if sufficient NaOH solution is added to give p_H about 7·5, and they are precipitated from solution at about p_H 4 to 4·5. The *p*-bromophenylureido-compounds of caseinogen and gelatin have been precipitated with alcohol *plus* acetic acid, washed with alcohol, then with an alcohol-ether mixture and finally extracted with ether for 200 hours. The products obtained were stable white preparations which were soluble at p_H 7·5, with the application of a little heat (up to 35°) in the case of the gelatin compound, and from these solutions the protein could be reprecipitated again with acetic acid. Phenylureido-gelatin and *p*-bromophenylureido-gelatin readily form gels.

II. Immunological investigations.

Immunisation. The phenylureido-compound of horse serum-globulin was prepared as described in the first part of this paper and the solution kept in the ice-chest. A short time before it was required for injection purposes, phenol was added to give a concentration of 0·25 % in the solution.

This solution was injected into 5 rabbits at weekly intervals, most of the injections being intraperitoneal, although a few of the earlier injections were

made intravenously. The amount of solution injected into each rabbit at each injection was such as to contain approximately 0·25 g. of the protein. The sera of the rabbits were tested 7 days after the third and subsequent injections. Three rabbits gave good antisera after the third injection, whereas the other 2 rabbits required 4 injections.

Precipitin tests. Two drops of the immune serum were added to 0·25 cc. of the diluted antigen solution in a small test-tube ($2'' \times \frac{1}{4}''$). The mixture was shaken and observed after it had been incubated at 37° for $\frac{1}{2}$ hr., 1 hr. and 2 hrs., and usually after a further 18 hrs. at room temperature (15–20°). Unless otherwise stated, the readings given in the Tables are those obtained after 1 hr. at 37°.

The various concentrations of antigen tested (1 : 20, 1 : 100, 1 : 500 and 1 : 2500) correspond to dilutions of a 5 % protein solution, *i.e.* the solutions used contained 0·25, 0·05, 0·01 and 0·002 % of protein respectively.

The extent of precipitin formation is recorded as follows (in increasing degrees of precipitation):

– (no reaction), f.tr. (faint trace), tr. (trace), ±, +, +±, ++, *etc.*

Preparation of phenylureido-amino-acid compounds.

These compounds were prepared by the action at room temperature of phenyl *iso*cyanate (1·3 mol.) on a solution of the amino-acid (1 mol.) containing NaOH (1 mol.). The products were separated by the addition of HCl and were recrystallised from hot water. The glycine and alanine compounds gave melting-points similar to those recorded by Paal [1894].

Preparation of phenylureido-compounds of chicken and rabbit serum-proteins.

These preparations were similar to those of the corresponding compound of horse serum-globulin described in the first part of this paper. The quantities used were 10 cc. of serum, 10 cc. of phosphate buffer (p_H 8·0), 1 cc. of 10 % NaCl and 0·4 cc. of phenyl *iso*cyanate.

Experimental.

The sera obtained by the injection of phenylureido-globulin (horse serum) into rabbits were tested against horse serum (and the separated horse serum-globulin) and against phenylureido-horse serum-globulin. Very powerful reactions were obtained with the last named antigen, indicating the possession of good antigenic power by the phenylureido-protein compound. With horse serum, or the separated horse serum-globulins, three of the antisera gave moderate and the other two very feeble or no reaction (*cf.* Table II).

These findings appear to indicate the loss, to some extent at least, of the original species-characteristics, especially since good reactions were obtained between antisera to phenylureido-horse serum-globulin and the phenylureido-compounds of chicken serum-proteins and similar compounds of rabbit serum-proteins; no reaction was obtained, of course, with untreated chicken or rabbit serum. Antisera to horse serum (or horse serum-globulin) gave good precipitin reactions with phenylureido-horse serum-globulin, so that in this respect the "horse" characteristics of the globulin have not been entirely lost as a result of the action of phenyl *iso*cyanate.

Further tests were made to determine whether these antisera to the phenyl *iso*cyanate compounds of horse serum-globulin reacted with other phenylureido-

Table II. *Precipitin reactions between anti-sera to phenylureido-horse serum-globulin and phenylureido-compounds of various serum proteins.*

		Immune serum against					
		Phenyl *iso*cyanate compounds of horse serum-globulin				Horse serum	Horse serum-globulin
Antigen	Dilution of antigen	No. 51	No. 52	No. 53	No. 54	No. 145	No. 43
Horse serum	1 : 20	±	tr.	−	−	++	++±
	1 : 100	+±	+	−	−	++	++
	1 : 500	+	tr.	tr.	−	+	+
	1 : 2500	tr.	tr.	f.tr.	f.tr.	tr.	tr.
Horse serum-globulin	1 : 20	+	tr.	−	−	++±	+++
	1 : 100	+±	+	f.tr.	−	++	++
	1 : 500	±	±	±	f.tr.	+	+
	1 : 2500	tr.	f.tr.	f.tr.	−	f.tr.	tr.
Phenyl *iso*cyanate of horse serum-globulin	1 : 20	+++	+++	++±	++	++±	+++
	1 : 100	++	++	+±	++	++	++
	1 : 500	+±	+±	+±	+±	±	+
	1 : 2500	+	+	+	+	tr.	tr.
Phenyl *iso*cyanate of chicken serum-proteins	1 : 20	+	++	+	+	−	−
	1 : 100	+±	+±	+±	+	−	−
	1 : 500	+	++	+	+	−	−
	1 : 2500	±	±	±	±	−	−
Phenyl *iso*cyanate of rabbit serum-proteins	1 : 20	+	+	±	+	−	−
	1 : 100	+	+±	+	+	−	−
	1 : 500	+±	+±	±	+	−	−
	1 : 2500	±	±	±	±	−	−

Note. The results with serum No. 50 are not included in the above Table since this serum and serum No. 51 gave practically identical results.

protein compounds, those of caseinogen and gelatin (*cf.* Tables III and IV). The results show that all these antisera gave good precipitin reactions with phenylureido- and *p*-bromophenylureido-caseinogen and also with similar compounds of gelatin, the results with the gelatin compounds being of special interest

Table III. *Precipitin reactions with various caseinogen compounds.*

		Immune serum against											
		Phenyl *iso*cyanate compounds of horse serum-globulin								Horse serum		Horse serum-globulin	
Antigen	Dilution of antigen	No. 50		No. 52		No. 53		No. 54		No. 145		No. 43	
Phenyl *iso*cyanate compound of chicken serum-proteins	1:20	±	±	+±	++	±	±	+	+	−	tr.	f.tr.	f.tr.
	1:100	+	+	+±	++	+	+	+	+	−	−	f.tr.	f.tr.
	1:500	+	+	+±	+±	±	+	+	+	−	−	−	−
	1:2500	±	±	±	+	±	±	±	±	−	−	−	−
Caseinogen	1:20	−	−	−	−	−	−	−	−	−	−	−	−
	1:100	−	−	−	−	−	−	−	−	−	−	−	f.tr.
	1:500	−	−	−	−	−	−	−	−	−	−	−	f.tr.
	1:2500	−	−	−	−	−	−	−	−	−	−	−	−
Caseinogen-phenyl *iso*cyanate compound	1:20	±	±	±	+	tr.	±	tr.	±	−	−	−	−
	1:100	+	+	+	+±	±	+	±	+	−	−	−	−
	1:500	+	+	+±	+±	+	+	+	+	−	−	−	−
	1:2500	+	+	+	+±	+	+	+	+±	−	−	−	−
Caseinogen-*p*-bromo-phenyl *iso*cyanate compound	1:20	±	±	±	±	−	f.tr.	±	±	−	−	−	−
	1:100	±	+	+	+±	tr.	±	±	±	−	f.tr.	f.tr.	f.tr.
	1:500	+	+	+	+±	±	+	+	+	−	−	f.tr.	f.tr.
	1:2500	tr.	±	tr.	±	±	±	±	±	−	−	−	−

Note. In this table, and in Table IV, the first reading is that obtained after the tubes had been incubated at 37° for half an hour, and the second reading is that made after one hour at 37°.

Table IV. *Precipitin reactions with phenylureido-gelatin compounds.*

		Immune serum against											
		Phenyl *iso*cyanate compounds of horse serum-globulin								Horse serum-globulin		Horse serum	
		No. 51		No. 52		No. 53		No. 54		No. 43		No. 145	
Antigen	Dilution of antigen												
Phenyl *iso*cyanate compound of chicken serum-proteins	1:20	+±	+±	++	++	+	+±	+	+	−	f.tr.	f.tr.	tr.
	1:100	+	+	+±	+±	+	+±	+	+	−	f.tr.	−	−
	1:500	+	+	+±	+±	±	+	+	+	−	−	−	−
	1:2500	+	+	+	+	+	+	+	+	−	−	−	−
Gelatin	1:20	−	−	−	−	−	−	−	−	−	−	−	−
	1:100	−	−	−	−	−	−	−	−	−	−	−	−
	1:500	−	−	−	−	−	−	−	−	−	−	−	−
	1:2500	−	−	−	−	−	−	−	−	−	−	−	−
Gelatin-phenyl *iso*cyanate compound	1:20	−	tr.	−	tr.	−	tr.	−	tr.	−	−	−	−
	1:100	−	±	f.tr.	±	−	f.tr.	tr.	f.tr.	−	−	−	−
	1:500	±	+	+±	+	±	±	+	+	−	−	−	−
	1:2500	+	+	+	+	+±	+	+±	+	−	−	−	−
Gelatin-*p*-bromo-phenyl *iso*cyanate compound	1:20	−	−	−	−	−	−	f.tr.	−	−	−	−	−
	1:100	tr.	f.tr.	tr.	±	tr.	tr.	tr.	tr.	−	−	−	−
	1:500	±	±	+	+	±	±	±	±	−	−	−	−
	1:2500	+	+	+	+	+	+	+	+	−	−	−	−

in view of the fact that gelatin itself is non-antigenic. The results given in these two Tables extend, therefore, the observations with the phenylureido-compounds of various serum-proteins, and they show that there exists a close immunological relationship between the phenylureido-compounds of serum-proteins and those of such widely different proteins as gelatin and caseinogen.

Finally, in order to obtain, if possible, confirmation of the view that the reaction between phenyl *iso*cyanate and proteins involves the free amino-groups of the protein molecule and to attempt to define more precisely the nature of the grouping responsible for the new immunological specificity, a few preliminary inhibition tests have been carried out. Phenylureido-compounds of glycine, alanine and lysine were prepared and the influence of these compounds and the untreated amino-acids on various precipitin reactions determined.

Table V. *Inhibition tests.*

0·20 cc. of *M*/100 solution of the "inhibiting" substance, *plus* 0·05 cc. of the antigen solution (1 : 20 or 1 : 100), *plus* 0·10 cc. of the antiserum.

			"Inhibiting" substance						
							Phenylureido-compounds of		
Antigen	Antiserum against	Antigen dilution	NaCl	Glycine	Alanine	Lysine	Glycine	Alanine	Lysine
Horse serum	Horse serum	1:20	++	++	++	++	++	++	++
		1:100	+±	+±	+±	+±	+±	+±	+±
Phenylureido-chicken serum-proteins	Phenylureido-horse serum-globulin	1:20	+±	+±	+±	+±	±	±	−
		1:100	+±	+±	+±	+±	tr.	tr.	−
Horse serum	Phenylureido-horse serum-globulin	1:20	+	+	+	+	+	+	+

The results, given in Table V, show that all these phenylureido-amino-acid compounds inhibit to a very marked extent, in the case of the lysine compound completely, the reaction between phenylureido-proteins and the corresponding antiserum, whereas these substances have no influence at all on the formation of precipitates in other antigen-antibody reactions (*e.g.* the precipitin reaction between horse serum-proteins and an immune serum against horse serum-

proteins). In the experiments recorded in Table V, the phenylureido-amino-acid compounds and the amino-acids were used in equimolecular amounts, but since there appeared to be a possibility that the greater influence of the phenylureido-lysine compound compared with the corresponding glycine and alanine derivatives might be due to the presence in the lysine compound of two phenylureido-groupings, similar tests were always made in addition with one-half that molecular concentration of the lysine compound. The smaller amount of phenylureido-lysine invariably gave complete inhibition of the "phenylureido" precipitin reaction.

Discussion.

The immunological investigations described here were mainly directed to the study of the effect of treatment with phenyl *iso*cyanate on the antigenic power and the specificity of certain protein antigens. In brief, the results of these studies (Tables II, III and IV) show that treatment with phenyl *iso*cyanate does not lead to any significant loss of the antigenic power of serum-globulin, but it does lead to a marked change in specificity. Thus, antibodies to the altered protein (phenylureido-horse serum-globulin) will react very well with the antigen used for the injection and with any similarly altered protein (phenylureido-chicken serum-proteins, phenylureido-caseinogen, phenylureido-gelatin, *etc.*) and to a moderate or very slight extent only with the protein which was used for the preparation of the phenylureido-protein injected (in this instance, horse serum-globulin). This chemical treatment has resulted, therefore, in a very pronounced alteration in immunological specificity, the old species-specificity being replaced by a specificity characteristic for the new type of protein used. That the old species-specificity is not completely lost, however, is shown by the fact that some of the antisera to the phenyl *iso*cyanate-protein compounds react with the unchanged homologous proteins, and by the good reaction between these phenyl *iso*cyanate-protein compounds (phenylureido-horse serum-globulin) and antisera to the unchanged protein (horse serum-globulin). This retention of some of the original species-specificity appears to indicate the presence in the altered protein molecule of some or all of those groupings or structures which normally determine species-specificity, and this might be due (*a*) to the fact that these groups are not acted upon or interfered with in any way by phenyl *iso*cyanate, or (*b*) to an incomplete reaction between the protein and phenyl *iso*cyanate. The second possibility has certainly a fair amount of experimental support, since the amino-N determinations (Table I) show that with the proteins used for the injections (horse serum-globulin and its phenylureido-derivative), the phenylureido-compound still retains about one-third of its free amino-N, from which it might be concluded that only two-thirds of the reactive groups of the protein molecule had combined with phenyl *iso*cyanate. Without further evidence, however, it would not be justifiable to conclude that the groups of the protein molecule which react with phenyl *iso*cyanate are concerned with the determination of species-specificity, but the evidence presented here seems to offer some support in favour of such a suggestion. It would be difficult, otherwise, to explain why some antisera to phenylureido-horse serum-globulin give a very faint or a negative reaction with unchanged horse serum-globulin, and the most reasonable conclusion is that the immunologically significant groups in the horse serum-globulin have been altered or destroyed by the phenyl *iso*cyanate. A possible alternative explanation, however, would be that the introduction of any new grouping into a protein molecule produces a new structural arrangement which is more prominent than are the other

groupings of the molecule in determining antibody formation when the new protein is injected into an animal. Thus the injection of phenylureido-horse serum-globulin would, according to this view, produce antibodies which are mainly directed against the phenylureido-grouping and would occasion no significant antibody response to the groupings which determine the specificity of horse serum-globulin, although these latter groupings may be unaltered by the treatment with phenyl *iso*cyanate.

Whichever explanation is adopted, however, and the weight of evidence appears to be in favour of the view that the groups acted upon by phenyl *iso*-cyanate are concerned in part at least with the original species-specificity, it is of importance to know the nature of the chemical reaction between phenyl *iso*cyanate and the protein molecule. From the evidence produced in the first part of this paper, principally relating to the diminution in free amino-N and the relationship between this reduction and the amount of bromine in the *p*-bromophenyl *iso*cyanate-protein compounds, the conclusion is reached that phenyl *iso*cyanate reacts with the free amino-groups of the protein molecule. The free amino-N of proteins represents, according to Van Slyke and Birchard [1913] and later authors, one-half of the lysine nitrogen, indicating that one amino-group only of the lysine molecule is bound. This would suggest that with phenyl *iso*cyanate the reaction takes place with the free amino-groups of the lysine, presumably the ϵ-amino groups, and from the chemical evidence presented here there is much support for this view. In addition to the evidence presented in the first part of this paper, it might be mentioned that attempts to prepare *p*-bromophenyl *iso*cyanate compounds of zein, a protein deficient in lysine, have been unsuccessful. Furthermore, the bromine contents of the *p*-bromophenyl *iso*cyanate compounds of caseinogen and gelatin usually represent 70 to 90 % of the theoretical values calculated from the figures given in the literature for the lysine contents of caseinogen and gelatin. The chemical evidence although suggestive is not conclusive, however, and it is left for serological inhibition tests to supply further support for the view that phenyl *iso*cyanate reacts with the free amino-groups of the lysine molecules.

Landsteiner [1920] showed that simple organic substances which could be coupled with proteins, although they showed no antigenic power themselves, were able to combine with the specific antibodies against the coupled proteins and could in this way prevent the formation of a precipitate when the coupled protein was added. Thus *m*-aminobenzoic acid added to an immune serum against *m*-aminobenzoic acid-azo-protein prevents the formation of a precipitate when *m*-aminobenzoic acid-azo-protein is added. These inhibition tests have since been used with success in Landsteiner's laboratory [Landsteiner and van der Scheer, 1928; 1931; 1932; Wormall, 1930] and by other authors [Klopstock and Selter, 1928; Avery and Goebel, 1929] and all the evidence available points to a marked specificity of this test. It is possible by means of this test to demonstrate inhibition by a substance with configuration identical with or very similar to that of the grouping attached to the protein molecule (Landsteiner; Landsteiner and van der Scheer) or it is possible to demonstrate the presence in a chemically altered protein of certain specific groupings, such as the 3 : 5-dihalogenated tyrosine groupings in iodinated and brominated proteins (Wormall). These inhibition tests have proved extremely useful in many of the investigations already quoted and it appears probable that they will in the future be of considerable assistance in studies on the chemistry of proteins by immunological methods. It is not proposed to discuss here at any length the significance of inhibition tests with the phenyl *iso*cyanate-protein precipitin reaction, but

it will perhaps be sufficient to say that such tests have served to confirm the view that the immunological specificity of these new protein compounds is due to the phenylureido-amino-acid groupings, in all probability the phenylureido-lysine grouping. As the preliminary results in Table V indicate, the precipitin reaction between phenylureido-proteins and antibodies to these protein compounds is not inhibited by certain amino-acids but is strongly inhibited by the corresponding phenylureido-amino-acid compounds, phenylureido-lysine being more effective than the corresponding glycine and alanine compounds. Other phenylureido-amino-acid compounds have still to be tested, but it appears justifiable to conclude from these early tests that the phenyl *iso*cyanate-protein compounds contain phenylureido-lysine groupings. Since this inhibition is so remarkably specific, and since phenylureido-lysine effects complete inhibition, it seems probable that no significant change, other than the formation of phenylureido-groupings, occurs when phenyl *iso*cyanate acts on proteins. If any other groups were introduced, or if any other significant change was effected in the protein molecule, the inhibition with phenylureido-lysine would probably be incomplete, since this compound has no inhibitory influence on the formation of other precipitates, such as that between horse serum-proteins and antisera to the phenyl *iso*cyanate compounds of horse serum-globulin, a reaction due to the retention in these phenyl *iso*cyanate-protein compounds of some of the horse specificity characteristics.

These immunological studies appear to suggest, therefore, that the lysine molecules of the protein are of some significance in determining species-specificity, and these conclusions would fall into line with those of Landsteiner and his co-workers, who have shown that changes which are not related to changes in the aromatic groupings of the protein molecule may destroy the species-specificity and may produce a specificity associated with the new group introduced; for example, acetylation, esterification and methylation result in a new specificity characteristic for the type of altered protein used [*cf.* reviews by Hartley, 1931; Landsteiner, 1927; 1933; Wells, 1929]. Formaldehyde has a somewhat different effect, since it converts rabbit serum-proteins into compounds which will produce in the rabbit antibodies to the antigen injected but not to formaldehyde-treated proteins of any other species [Landsteiner and Jablons, 1914]. There is perhaps sufficient evidence that the aromatic groupings of the protein are concerned in some way with specificity (for a review of the literature *cf.* Wormall [1930]), and yet there is quite as much or even more support for the view that non-aromatic groupings determine species-specificity. It may be that the introduction of any new grouping, if the group is sufficiently prominent, will lead to the acquirement of a new immunological specificity characteristic for the group introduced. With reference to the question of the significance of lysine, it is of interest to note that for a protein to be fully antigenic it is not necessary for it to contain lysine, since zein and gliadin contain no significant amount of lysine, but have marked antigenic powers. Thus all types of protein specificity cannot be related to the lysine groups.

The results of the experiments carried out with the phenyl *iso*cyanate and *p*-bromophenyl *iso*cyanate compounds of gelatin (Table IV) show that these compounds readily give precipitates with antisera to phenylureido-horse serum-globulin. This appears to be of special interest in view of the non-antigenicity of gelatin, and further investigations are being carried out to determine whether the introduction of aromatic groups into the gelatin molecule in this manner leads to the development of full antigenic power. Phenylureido- and *p*-bromophenylureido-gelatin compounds are being injected into rabbits, and tests are

being made to determine whether antibody production occurs. The results of these experiments will be reported later. Adant [1930] has succeeded in obtaining precipitin reactions with suitable antisera and gelatin coupled with diazotised aniline, and Medveczky and Uhrovits [1931] have obtained similar results with benzoylated gelatin. Adant reports that the gelatin-diazotised aniline complex is fully antigenic.

The results of the immunological tests with the *p*-bromophenylureido-proteins are almost identical with those with the phenylureido-compounds, and antibodies to the latter readily react with the former, although the intensity of the reaction may not always be quite so strong. This result is perhaps what one would expect, the introduction of a bromine atom effecting a difference in the new grouping which does not alter the specificity but reduces somewhat the intensity of the reaction. These observations support, therefore, the conclusions of Landsteiner and van der Scheer [1927], who found that the specificity of azo-proteins is not necessarily greatly modified by the introduction into the aromatic nucleus of the diazo-compound of various groups, such as CH_3, NO_2, Br, Cl, *etc.*

In conclusion, it appears from the results already obtained with the phenyl *iso*cyanate-protein compounds used in the work described here that this type of compound will be of some considerable use in the study of the protein molecule from the immunological point of view. For this purpose, this method of altering the molecule or introducing new groupings has certain very definite advantages. It is relatively rapid and is not very drastic, since it can be carried out at low temperatures and in neutral or practically neutral solution. Furthermore, the excess of the reagent reacts with water to give an insoluble substance which can readily be removed.

Summary.

1. Phenylureido- and *p*-bromophenylureido-compounds of caseinogen, gelatin and horse serum-globulin have been prepared by the action of phenyl *iso*cyanate and *p*-bromophenyl *iso*cyanate on solutions of these proteins kept at p_H 7 to 8. The properties of these compounds are described.

2. Evidence is presented which suggests that phenyl *iso*cyanate and *p*-bromophenyl *iso*cyanate react only with the free amino-groups of the lysine molecules of the protein.

3. The phenylureido-derivative of horse serum-globulin, when injected into rabbits, produces antibodies which give precipitin reactions with the phenylureido- and *p*-bromophenylureido-compounds of horse serum-, chicken serum- or even rabbit serum-proteins, and with similar compounds of caseinogen and gelatin. Thus, antibodies capable of reacting with any phenylureido-protein are produced.

4. The antisera to phenylureido-horse serum-globulin do not react very strongly with the unchanged globulin; indeed, with one immune serum of this type, no precipitate at all was given with horse serum-globulin. Thus there appears to be a considerable loss, when the serum-proteins are converted into the corresponding phenylureido-compounds, of the original species-specificity or characteristics, although antisera to native horse serum-globulin give very marked precipitates with phenylureido-horse serum-globulin. The significance of these findings is discussed.

5. The precipitin reaction between phenylureido-proteins and the antisera to these compounds is specifically inhibited by phenylureido-amino-acid compounds (glycine, alanine, and lysine). Inhibition is complete with the lysine

compound and not quite complete with the other phenylureido-compounds tested. This supports the view that the characteristic grouping in the new phenylureido-proteins is phenylureido-lysine.

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