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

II. THE GELATIN COMPOUNDS.

By SYDNEY JOHN HOPKINS AND ARTHUR WORMALL.

From the Department of Physiology, the University of Leeds.

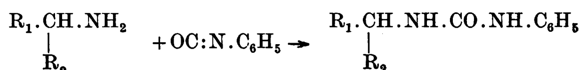
(*Received August 31st, 1933.*)

NATIVE proteins, with a few exceptions, when injected into the animal body readily produce antibodies. By a comparison of the chemical constitution and properties of various proteins it is possible to say that the presence of a certain amino-acid or grouping is not essential for antigenic power, and, in some instances, to offer a feasible explanation for the non-antigenicity of a certain protein. Thus, although zein is deficient in tryptophan and lysine, and caseinogen in cystine, these two proteins are markedly antigenic, and the conclusion can be reached, therefore, that the three amino-acids mentioned are not essential for antigenicity. The protamines [Wells, 1913; *cf.* review by Wells, 1929], gelatin [Wells, 1908; 1916; Landsteiner, 1917; Starin, 1918; Kahn and McNeil, 1918] and the closely related preparation, glue [Ramsdell and Walzer, 1927], on the other hand, are non-antigenic. Gelatin appears to be of special interest in this respect, and the non-antigenicity of this protein has attracted much attention. Starin [1918], in particular, carried out an extensive investigation, using the precipitin, anaphylactic, complement fixation and meiostagmin reactions, and decided that the injection of gelatin into rabbits, guinea-pigs and dogs failed to produce antibodies to gelatin. This failure of gelatin to incite antibody production has been interpreted in several ways, but the view most commonly held suggests that the non-antigenicity in this instance is due to the absence of aromatic groupings, for gelatin is deficient in tyrosine and tryptophan, and it contains only a very small amount of phenylalanine. Gelatin, however, differs from other proteins in many respects, and several additional suggestions might be advanced to account for its peculiar immunological properties. Thus, it is important to know whether the failure to produce antibodies is entirely due to the absence of aromatic groupings, and for this purpose it was decided to introduce into the gelatin molecule groupings of this nature.

Landsteiner [1919] found that gelatin coupled with diazotised metanilic acid gave precipitin reactions with antisera produced by the injection of horse-serum-proteins coupled with *m*-diazobenzenesulphonic acid, and that in higher concentrations the gelatin compound specifically inhibited the precipitin reaction between these azoproteins and their antisera. Adant [1930] and Bruynoghe and Vassiliadis [1930] studied the serological properties of gelatin coupled with diazotised aniline and obtained some antibody formation when this preparation was injected. The results of these authors will be discussed more fully later on in this paper. Medveczky and Uhrovits [1931], working with benzoylated proteins, have shown that immune sera produced by the injection of the benzoylated

proteins of horse serum or of benzoylated typhoid bacilli will give good precipitin reactions with benzoylated gelatin, and that the injection of benzoylated gelatin produces anaphylaxis in animals sensitised with benzoylated typhoid bacilli. Hooker and Boyd [1933] have very recently studied the immunological properties of gelatin coupled with diazotised arsanilic acid, and have found that the injection of this preparation produces sera which give precipitin and complement fixation reactions with other proteins coupled with diazotised arsanilic acid but not with the homologous antigen (gelatin-diazo-arsanilic acid). The evidence so far available appears to show, therefore, that gelatin coupled with these new groupings can take part in precipitin and similar reactions, but evidence of the acquirement of true antigenic function does not appear to be very satisfactory.

In a previous paper [Hopkins and Wormall, 1933] the immunological and chemical properties of phenylureidoproteins have been studied. These compounds are prepared by the action of phenyl *iso*cyanates on various proteins, a reaction used by Raper [1907] for the preparation of phenylureidopeptone compounds. By this reaction, a new grouping, $C_6H_5NH.CO$— or a related group, is introduced into the protein molecule, and in the earlier paper evidence has been presented which strongly suggests that the introduction occurs mainly, if not entirely, at the free amino-groups of the lysine molecules. The reaction can be represented in the following manner:



In this way, aromatic groupings are introduced into the protein, and the mode of linkage is not very different from the ordinary peptide linkage which appears to link up most of the amino-acids in the protein molecule. This reaction thus offers an alternative method for studying the effect of the introduction of new groups into the protein molecule, and for several reasons it is most suitable for this purpose. The mode of linkage is not unlike that already present and the protein is not subjected to very drastic treatment. Furthermore, it appears very desirable that different methods of introducing the same new group into different parts of the same protein molecule might be studied. Experiments along these lines are being carried out and will be reported later. In the investigation described here, a study has been made of the chemical and immunological properties of phenylureido- and *p*-bromophenylureido-gelatin, the primary object being to determine whether the introduction of aromatic groupings into the gelatin molecule would render it fully antigenic. For this purpose these gelatin compounds have been tested, in precipitin and complement fixation tests, with several antisera against phenylureido-horse-serum-globulin, and immunisation experiments have been carried out to determine whether antibodies are produced when these phenylureidogelatin compounds are injected into rabbits.

EXPERIMENTAL.

Preparation of phenylureidogelatin.

4 g. of gelatin (Coignet's "Gold Label") were dissolved in 400 cc. of warm water, 200 cc. of a phosphate buffer of p_H 8·0 added, the mixture cooled and stirred and 3·2 cc. of phenyl *iso*cyanate added. The mixture was kept at p_H 8 and stirred for about 1 hour. The phenylureidogelatin was then precipitated by acetic acid and purified by two further precipitations as described in a previous

paper [Hopkins and Wormall, 1933]. This amount of gelatin usually furnished under these conditions about 60–80 cc. of a solution containing about 2 % of protein. NaCl was added to give 0·9 %, and the solution was filtered through a Berkefeld filter-candle. The filtered solution was kept in sterile tubes in the ice-chest, and the solidified mass was melted by gentle heat when required.

The solutions thus obtained were perfectly clear and closely resembled solutions of gelatin. The phenylureidogelatin compounds, like the similar compounds of serum-globulin and caseinogen, can be precipitated by the addition of dilute acetic acid to p_H 4–4·5, and the precipitates are readily soluble again at p_H 7·5, although with the gelatin preparations dissolution is best effected by warm water or NaCl solution (25–30°). The solutions used in this work (containing 1–2·5 % of phenylureidogelatin) solidified on cooling, and thus substitution in the free amino-groups of the gelatin molecule does not destroy the capacity to form gels. In this connection, it is interesting to note that gelatin treated with nitrous acid or with formaldehyde still exhibits gel-formation.

Preparation of p-*bromophenylureidogelatin.*

This compound was prepared by the addition of a filtered ethereal solution of *p*-bromophenyl *iso*cyanate (4 g. in about 30 cc. of ether) to a cooled and stirred mixture of 400 cc. of 1 % gelatin (Coignet's "Gold Label") and 200 cc. of phosphate buffer of p_H 8·0. The mixture was cooled, kept at p_H 8 and stirred for about 1½ hours, after which precipitation and purification were effected as described previously [Hopkins and Wormall, 1933]. From this amount of gelatin, 50–60 cc. of a solution containing about 2·5 % of *p*-bromophenylureidogelatin were usually obtained. NaCl was added to the extent of 0·9 % and the solution filtered through a Berkefeld filter-candle and kept in the ice-chest.

Nitrogen determinations.

The ratios of free amino-N to total N in the above preparations and in gelatin were determined, and in this way a measure of the amount of substitution was obtained, as described in the previous paper. The main bulk of the antigen solutions used for the immunisation experiments gave the following figures for the free amino-N (Sørensen's formaldehyde method) calculated as a percentage of the total N: gelatin, 3·0 %; phenylureidogelatin, 0·8 % and *p*-bromophenylureidogelatin 1·2 %. These figures indicate substitution to the extent of 70–75 % in the phenylureido-compound and about 60 % in the *p*-bromophenylureidogelatin. The last-named compound was found to contain 2·2 % of bromine, whilst another preparation of the same substance had a bromine content of 2·6 %.

Immunisation.

Three groups, each of 4 rabbits, were used for the injection of the following substances:

Group (*a*) Gelatin (Coignet's "Gold Label").
Group (*b*) Phenylureidogelatin.
Group (*c*) *p*-Bromophenylureidogelatin.

The control injections with untreated gelatin were made in order to establish the non-antigenicity of the sample of gelatin used in this investigation. The two phenylureidogelatin compounds were prepared as described above. It was thought desirable in these experiments, where the introduction of aromatic

groupings was being studied, to avoid the use of phenol as antiseptic, and therefore all the solutions used for injection were sterilised by filtration through Berkefeld filter-candles.

At each injection the rabbits received a volume of the antigen solution containing 0·25 g. of the protein, and the injections were made at intervals of 7 or 8 days. These injections were usually made intraperitoneally, but with 50 % of the animals the last two injections were intravenous. Samples of serum from each rabbit were tested 7 or 8 days after the third, fourth, fifth, sixth and seventh injections and also about 3 weeks after the seventh injection. Precipitin tests were carried out on each occasion, and usually complement fixation tests were also made. Each serum was tested against all three antigens (gelatin, phenylureidogelatin and *p*-bromophenylureidogelatin) and against phenylureido-chicken-serum-proteins and phenylureido-rabbit-serum-proteins.

Precipitin tests. The technique of these tests was as described previously [Johnson and Wormall, 1930; Hopkins and Wormall, 1933]. Some of the tests were made at 37°, but the majority were done at room temperature (15–20°), since it was found early in this work that the gelatin antigens gave much better precipitin reactions at the lower temperatures. In many instances tests were made at 37° as well as at room temperature. The results were recorded as follows: (no reaction), f.tr. (faint trace), tr. (trace), ±, +, +±, ++ (increasing degrees of precipitation).

Complement fixation tests. These were carried out as described previously [Johnson and Wormall, 1930], the results being recorded as follows: 4 (complete haemolysis), – (no haemolysis), 3, 2 and 1 (intermediate degrees of haemolysis).

(1) *The phenylureidogelatin compounds and their reactions with antisera to phenylureido-horse-serum-globulin.*

The preliminary observation that phenylureidogelatin preparations give precipitates with antisera to phenylureido-horse-serum-globulin, noted in a previous paper [Hopkins and Wormall, 1933], was amplified by making tests with various antisera and using much wider ranges of antigen dilution. These precipitin reactions were also compared with those obtained in similar series of phenylureido-horse-serum-proteins and phenylureido-chicken-serum-proteins tested against the same antisera. Precipitin tests were made both at 37° and at room temperature, and from the results obtained (*cf.* Table I) the conclusion was reached that room temperature is preferable to 37° for precipitin tests with the gelatin antigens. Indeed it has frequently been observed that well-marked precipitates of phenylureidogelatin *plus* antisera to phenylureido-horse-serum-globulin have become much less bulky after incubation at 37°. This was well illustrated in the experiment quoted in Table I, for on the addition of the antiserum to phenylureidogelatin good precipitates were obtained, and these became much less pronounced after 1 hour at 37° and still less marked after 3 hours at this temperature. When these antigen-antibody mixtures at 37° were cooled, the gelatin antigen precipitates became stronger again and were comparable with those at room temperature. Thus the precipitates of "phenylureido"-antiserum with phenylureidogelatin appear to be more soluble at 37°, possibly owing to the retention by the gelatin of some of its solubility properties. The other antigens used in these tests, phenylureido-chicken-serum-proteins and phenylureido-horse-serum-proteins behaved in the normal manner and with antisera to phenylureido-proteins gave precipitates at 37° which were equal to, or greater than, those obtained at room temperatures.

Table I. *Comparison of the precipitin reactions with various phenylureido-protein compounds and the influence of temperature on these reactions.*

Antigen	Antigen dilution	Immune serum (anti-phenylureido-horse-serum-globulin) No. 50 16°	No. 50 37°	No. 51 16°	No. 51 37°	No. 53 16°	No. 53 37°
Phenylureidogelatin	1:20	–	–	–	–	–	–
	1:100	f.tr.	–	tr.	f.tr.	tr.	–
	1:500	±	tr.	+	±	+	±
	1:2500	+	±	+	±	+	±
	1:12,500	tr.	tr.	tr.	tr.	tr.	tr.
Phenylureido-chicken-serum-proteins	1:20	±	±	+	+	±	+
	1:100	+	+	+	+	+	+
	1:500	+	+	+	+ ±	±	±
	1:2500	tr.	tr.	±	±	tr.	tr.
	1:12,500	–	–	–	–	–	–
Phenylureido-horse-serum-proteins	1:20	+ +	+ +	+ + ±	+ + ±	+ ±	+ ±
	1:100	+ + ±	+ + ±	+ + ±	+ + ±	+ +	+ +
	1:500	+ ±	+ ±	+ ±	+ ±	+	+ ±
	1:2500	±	±	±	±	tr.	±
	1:12,500	–	–	–	f.tr.	–	–

The results given in Table I indicate a very marked capacity on the part of phenylureidogelatin to give precipitates with antisera to phenylureido-horse-serum-globulin, but maximum precipitation is observed with much greater dilutions of the gelatin preparations compared with the similar preparations of horse-serum-proteins or chicken-serum-proteins. These differences in the zone of maximum precipitation may be related to the number of reactive groupings in the antigen, but this does not appear probable since the preparations used for these tests (Table I) did not differ to any great extent in the number of phenylureido groupings introduced. Complement fixation tests have also demonstrated the same difference in capacity to react with the antiserum, the phenylureidogelatin preparations being active in much higher dilutions than are the corresponding serum-protein compounds (*cf.* Table II).

Table II. *Complement fixation tests with phenylureidogelatin compounds.*

(Tests with immune serum, No. 51—anti-phenylureido-horse-serum-globulin.)

Antigen	Antigen dilution 1:20	1:60	1:180	1:540	1:1620	1:4860	1:14,580	1:43,740	Control (NaCl)	Reading after (hr.)
Phenylureido-horse-serum-proteins	4	3	—	—	—	—	1	4	4	½
	4	3	1	—	—	—	2	4	4	1
Phenylureido-gelatin	4	2	—	—	—	—	—	—	4	½
	4	4	1	—	—	—	—	—	4	1

The other gelatin preparation used in this investigation, *p*-bromophenylureidogelatin, gave very similar results with all the antisera, but the precipitin and complement-fixation reactions with this preparation were not quite so pronounced as those with phenylureidogelatin.

Inhibition tests, involving the addition of simple substances to mixtures of the antigen and antibody in order to determine which groupings could specifically inhibit the formation of the precipitate [Landsteiner, 1920; Landsteiner and van der Scheer, 1931; 1932] were made with certain amino-acids and their

phenylureido-compounds in a previous paper [Hopkins and Wormall, 1933]. These experiments, made with phenylureido-chicken-serum-proteins and antisera to phenylureido-horse-serum-globulin, furnished very strong evidence for the view that the immunologically active grouping in the phenylureidoprotein is the phenylureidolysine group. Similar results have been obtained from tests carried out with phenylureido- and *p*-bromophenylureido-gelatin as antigens in the precipitin reaction. Experiments have also been made to determine whether similar inhibition might be obtained in complement fixation tests with the phenylureidogelatin compounds, and the results of an experiment of this nature are given in Table III. From these results it will be seen that phenylureidolysine, and to a lesser extent phenylureidoalanine, inhibit these complement fixation reactions.

Table III. *Inhibition of complement fixation tests.*

Antiserum. No. 51 (anti-phenylureido-horse-serum-globulin).
Antigen. Phenylureidogelatin.

Antigen dilution	Inhibiting substance									
	NaCl		Lysine		Alanine		Phenylureido-lysine		Phenylureido-alanine	
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	2	3	3	4	2	3	4	4	4	4
1 : 540	—	—	—	2	—	—	4	4	3	4
1 : 1620	—	—	—	—	—	—	4	4	1	2
1 : 4860	—	—	—	—	—	—	4	4	—	1
1 : 14,580	—	—	—	—	—	—	4	4	—	2
1 : 43,740	—	—	—	—	—	—	4	4	2	3
1 : 131,220	1	3	1	2	1	3	4	4	4	4
1 : 393,660	3	4	2	3	3	4	4	4	4	4
Control (NaCl)	4	4	4	4	4	4	4	4	4	4
Reading (hr.) after	½	1	½	1	½	1	½	1	½	1

Details of tests. A mixture of 0·5 cc. of the antigen, 0·10 cc. of the inactivated antiserum, 0·10 cc. of 1 : 5 guinea-pig serum and 0·10 cc. of a neutral $M/100$ solution of the "inhibiting" substance (in 0·9 % NaCl solution) was kept at room temperature for 1 hour. 0·5 cc. of a 4 % suspension of sensitised ox red cells was then added to each tube and the tubes placed in a water-bath at 37°.

(2) *Immunisation experiments with phenylureido- and* p*-bromophenylureido-gelatin.*

In order to determine whether the introduction of aromatic groupings into gelatin really endows this protein with antigenic power, immunisation experiments have been carried out with phenylureidogelatin and *p*-bromophenylureidogelatin. In these experiments the modified gelatins were injected into rabbits as described earlier in this paper, and the sera of these rabbits were tested frequently by precipitin and complement fixation tests, for the presence of antibodies to gelatin itself, to phenylureidogelatin or to any other phenylureidoprotein (phenylureido-chicken-serum-proteins, *etc.*). Each rabbit received seven injections of the phenylureido- or *p*-bromophenylureido-gelatin, at intervals of 7 or 8 days, and the sera were tested 7 or 8 days after the third and subsequent injections. Throughout the whole of each series of injections the serological tests gave negative results, although there were one or two occasions when the precipitin tests made with these sera and phenylureido-chicken-serum-proteins (or phenylureido-rabbit-serum-proteins) showed very faint traces of a precipitate. These turbidities were very slight, however, certainly not more

significant than a faint trace, and they were only observed in very strong antigen solutions (a 1 : 20 solution of the 5 % protein solution). Since even these slight reactions were never obtained with the concentrations of antigen normally used for the testing of antisera, and since they were obtained on a few occasions only, the conclusion was reached that they are not significant. The complement fixation tests made simultaneously with the same sera and the same antigen solutions were completely negative on every occasion.

Discussion.

The action of phenyl *iso*cyanate and *p*-bromophenyl *iso*cyanate on gelatin results in the production of phenylureidogelatin compounds which readily give precipitin and complement fixation reactions with antisera to phenylureido-horse-serum-globulin. In the complement fixation tests these gelatin compounds appear to behave very much like the corresponding serum-protein compounds, but in the precipitin reactions they show certain significant differences. The chief difference is related to the influence of temperature on the extent of precipitation, the amount of precipitate formed with the gelatin compound being much less at 37° than at 16–20°. This difference is possibly related to the greater solubility of gelatin at the higher temperature. A comparison of the precipitin and complement fixation tests with phenylureidogelatin and the corresponding serum-protein compounds has also shown that the zone of maximum precipitation, or the zone of complete complement fixation, is found in much more dilute solution with the gelatin compounds. This difference in activity does not appear to be related to the number of phenylureidolysine groupings in the phenylureido-protein, and at the present time no explanation can be advanced to account for the greater power of the gelatin compounds to give precipitates in very dilute solution.

This ability to give precipitates or complement fixation when mixed with various antisera does not in any way prove that the phenylureidogelatin preparations are truly antigenic. A similar power to give precipitation with suitable antisera is exhibited by the soluble specific substances, which do not normally incite antibody response when injected into an animal [Heidelberger and Avery, 1923, *cf.* Review by Heidelberger, 1927]. More recent work suggests that some modification of the view that these immunologically active carbohydrates and similar haptens are non-antigenic, will perhaps be necessary, since it has been shown that certain of these haptens can produce antibodies when injected after adsorption on kaolin, collodion particles, charcoal, *etc.* [Gonzalez and Armangue, 1931; Zozaya, 1931; 1932; Landsteiner and Jacobs, 1932; 1933]. Any modification of this view does not affect, however, the argument advanced here that the capacity to give a precipitate or to give complement fixation when certain antisera are added does not necessarily imply that the substance is antigenic, *i.e.* that it will produce antibodies when injected into an animal. There are several other instances of precipitin reactions with substances which are not truly antigenic. Thus, antibodies to azoproteins will give precipitates with azo-dyes [Landsteiner and van der Scheer, 1932], indicating the specific combination of antibodies with substances of relatively small molecular weight to give a precipitate. The demonstration of true antigenic power involves the formation of antibodies when the substance is injected into an animal, these antibodies being detected by the precipitin, complement fixation or some similar test.

The immunisation experiments with phenylureido- and *p*-bromophenyl-ureido-gelatin compounds described in this paper have shown that these com-

pounds do not produce any significant antibody response detectable by precipitin or complement fixation reactions. From these results the conclusion can be reached, therefore, that to render gelatin antigenic in the full sense it is not sufficient to introduce aromatic groupings. The non-antigenicity of gelatin, although it might conceivably be due in part to the absence of such groupings, is not due solely to this deficiency. For several reasons it would be desirable to introduce into the gelatin molecule an aromatic grouping which is completely or almost completely identical with one of those present in other proteins, *e.g.* the tyrosine or tryptophan groupings linked to the rest of the molecule by means of a peptide linkage. It is hoped that experiments along these lines will be made in the near future, but it is realised that it may be extremely difficult to effect the introduction of a grouping of this kind without recourse to a fairly drastic method which will involve some other change in the protein molecule.

The results obtained by Adant [1930] and Bruynoghe and Vassiliadis [1930] are difficult to correlate with those recorded in this paper. Adant found that gelatin coupled with diazotised aniline produced, when injected into the rabbit, antibodies which would give precipitates with gelatin-diazotised aniline and also with gelatin itself. The reaction between these antisera and gelatin is most difficult to explain, and it appears necessary to postulate the acquirement of antigenic power when diazotised aniline is coupled with gelatin and the formation of at least two types of antibody or antibody groupings when this complex is injected; one antibody which can specifically react with any diazotised aniline-protein compound and another which can specifically react with gelatin. Bruynoghe and Vassiliadis [1930] carried out complement fixation tests with these antisera to gelatin-diazotised aniline and obtained fixation with gelatin and not with gelatin-diazotised aniline. These findings also are difficult to interpret and without further experimental details it would be impossible to assess the significance of these complement-fixation results. Hooker and Boyd [1933] have recently carried out similar investigations using gelatin-diazotised arsanilic acid, and they find that this gelatin compound produces in the rabbit antibodies which will give precipitin reactions with other proteins coupled with diazotised arsanilic acid but not with gelatin-diazotised arsanilic acid or with gelatin. These results of Hooker and Boyd indicate that antigenicity is to some extent conferred on gelatin by coupling it with diazotised arsanilic acid, but, as these authors point out, the results "do not show that the non-antigenicity of gelatin is due solely to its deficiency in aromatic amino-acids." Hooker and Boyd record the significant finding, but one which is difficult to interpret, that gelatin-diazotised arsanilic acid reacts with antisera to egg-white-diazotised arsanilic acid but not with antisera to gelatin-diazotised arsanilic acid, although the last-named antisera give precipitates with egg-white proteins (or caseinogen) coupled with diazotised arsanilic acid. From many points of view, it might be claimed that the method used in the work described here has several advantages over other methods which have been used for the introduction of aromatic groupings into the gelatin molecule. With the phenyl *iso*cyanate method, the linkage is one which does not differ very considerably from the ordinary peptide linkage, and a second point in its favour is that any excess of phenyl *iso*cyanate is rapidly destroyed and the products formed from it are easily removed from the phenylureidogelatin solution.

From the immunisation experiments described here, it appears probable that some explanation other than that relating to aromatic groupings will have to be advanced to account for the non-antigenicity of gelatin, but any suggestion offered at the present time will be mainly speculative. The chemical constitutions

of gelatin and other proteins can be compared and the non-antigenicity of the former attributed to the absence of some substance or grouping which is present in all antigenic proteins. Gelatin is deficient in, amongst other things, aromatic amino-acids and carbohydrate groupings. The mere introduction of benzene rings does not appear to convert gelatin into an antigen, and with reference to the second possibility there appears to be little or no evidence that proteins must contain a carbohydrate grouping before they are antigenic. The carbohydrates associated with certain bacterial proteins undoubtedly play the predominant rôle in the determination of certain types of specificity, and there are probably very many instances of specificity determined by the carbohydrates present. It does not necessarily follow, however, that protein specificity is always determined by the carbohydrate groupings which are usually associated with native proteins, nor does it mean that antigenicity is exhibited only by those proteins which contain carbohydrate groupings. In this case, however, as with the other suggestions which might be offered to account for the non-antigenicity of gelatin, it does not appear profitable at the present time to discuss the matter at any length, and much additional evidence will be needed before it is possible to reach any final decision.

SUMMARY.

1. Phenylureido- and *p*-bromophenylureido-gelatin have been prepared by the method previously described. Some properties of these compounds are described.

2. These gelatin preparations give marked precipitin and complement fixation reactions with antisera to phenylureido-horse-serum-globulin.

3. With the gelatin preparations the zone of maximum precipitation is found in higher dilutions than are the corresponding zones for phenylureido-compounds of horse-serum-globulin or of caseinogen. This difference does not appear to be related to the number of new groupings introduced.

4. The precipitin reactions with these gelatin compounds are most pronounced when carried out at room temperature (15–20°) or at lower temperatures, since there is a strong tendency for the precipitates to go into solution at 37°.

5. Phenylureido- and *p*-bromophenylureido-gelatin when injected into rabbits over a long period fail to produce antibodies which are detectable by precipitin and complement fixation tests. The conclusion is reached, therefore, that the non-antigenicity of gelatin is not due solely to a deficiency in aromatic groupings.

One of the authors (A. W.) is indebted to the Medical Research Council for a part-time personal grant and for a grant which has, in part, defrayed the expenses of this research.

REFERENCES.

Adant (1930). *Compt. Rend. Soc. Biol.* **103**, 541.
Bruynoghe and Vassiliadis (1930). *Compt. Rend. Soc. Biol.* **103**, 543.
Gonzalez and Armangue (1931). *Compt. Rend. Soc. Biol.* **106**, 1006.
Heidelberger (1927). *Physiol. Rev.* **7**, 107.
—— and Avery (1923). *J. Exp. Med.* **38**, 73; *cf.* also **38**, 81; **40**, 301, *etc.*
Hooker and Boyd (1933). *J. Immunol.* **24**, 141.
Hopkins and Wormall (1933). *Biochem. J.* **27**, 740.

Johnson and Wormall (1930). *Biochem. J.* **26**, 1202.
Kahn and McNeil (1918). *J. Immunol.* **3**, 277.
Landsteiner (1917). *Z. Immunitätsforsch.* **26**, 152.
—— (1919). *Biochem. Z.* **93**, 106.
—— (1920). *Biochem. Z.* **104**, 280.
—— and Jacobs (1932). *Proc. Soc. Exp. Biol. Med.* **29**, 570.
—— —— (1933). *Proc. Soc. Exp. Biol. Med.* **30**, 1055.
—— and van der Scheer (1931). *J. Exp. Med.* **54**, 295.
—— —— (1932). *Proc. Soc. Exp. Biol. Med.* **29**, 747; *J. Exp. Med.* **56**, 399.
Medveczky and Uhrovits (1931). *Z. Immunitätsforsch.* **72**, 256.
Ramsdell and Walzer (1927). *J. Immunol.* **14**, 207.
Raper (1907). *Hofmeister's Beiträge*, **9**, 168.
Starin (1918). *J. Infect. Dis.* **23**, 139.
Wells (1908). *J. Infect. Dis.* **5**, 449.
—— (1913). *Z. Immunitätsforsch.* **19**, 599.
—— (1916). *J. Biol. Chem.* **28**, 11.
—— (1929). The chemical aspects of immunity. (The Chemical Catalog Co., New York.)
Zozaya (1931). *Science*, **74**, 270.
—— (1932). *J. Exp. Med.* **55**, 325.