ON THE INFLUENCE OF ACID GROUPS ON THE SEROLOGICAL SPECIFICITY OF AZOPROTEINS.*

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PLATE 31.

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In previous work (2–5) investigations were made of the relation between chemical constitution and serological specificity by compounding various substances of simple chemical structure with proteins and using the resulting combinations as antigens. The coupling of proteins with diazonium derivatives of aromatic substances offered itself as the most suitable means. The immune sera obtained were tested by the precipitin reaction. In order to avoid species-specific reactions on the protein component the test antigens generally were prepared with a protein from another species than that employed for the immunizing antigens. The principal result of these studies was the observation that the immune sera exhibit a specificity which is determined by the chemical structure of the simple compounds attached to the protein.[1]

Aside from the phases already dwelt upon there are others to which the method outlined is applicable. The present paper deals with the influence of acid groups on specificity. Reference has been made to this question in one of the papers quoted (3).[2] For our purpose antigens were prepared with chemicals with and without acid groups; after immunization with these substances the immune sera were tested with both types of antigens.[3]

* Twenty-second paper on antigens and specificity. *Cf.* Reference 1.

[1] For a fuller review we refer to "The chemical aspects of immunity," by H. Gideon Wells, American Chemical Society Monograph Series, New York, 1925, page 77.

[2] Page 387.

[3] For the sake of brevity these will be referred to in the following as acid and non-acid antigens and immune sera respectively.

EXPERIMENTAL.

Preparation of the Antigens for Immunization.—As an example the method used for aniline is given. The other substances were taken in quantities equivalent to that of the aniline.

1.2 gm. of aniline were dissolved in 10 cc. of water and 5 cc. of 7 N HCl and diazotized with the necessary amount of sodium nitrite at a temperature of 0–5°C., with starch iodide paper as indicator. The diazo solution was diluted with ice water to a volume of 100 cc. and poured into a mixture of 100 cc. of horse serum and 100 cc. of normal sodium carbonate. The solution must give a strongly alkaline reaction with phenolphthalein. Coupling was allowed to take place for 10 minutes at 0–5°C. By acidification with hydrochloric acid the azoprotein was precipitated and after filtration it was suspended in a small amount of water. On addition of some normal sodium hydroxide and vigorous stirring it became viscous or jelly-like. A large quantity of alcohol was added and subsequently enough hydrochloric acid to flocculate the material.

The precipitate was filtered and treated twice again in the same manner. In order to remove the alcohol it was brought into solution with alkali as before and after dilution it was reprecipitated with hydrochloric acid and filtered. After grinding in a mortar the substance was dissolved in water at alkaline reaction. Finally the reaction was adjusted with hydrochloric acid to faint alkalinity or neutrality.

The volume was made up to 190 cc. with distilled water and the necessary amount of a salt solution to make the ultimate salt concentration approximately 1 per cent. 10 cc. of a 5 per cent phenol solution were added. The presence of some suspended azoprotein in the solution does not affect its use for the immunization.

The other preparations were made in a similar way. Some of them such as the azoproteins from ortho- and para-chloroaniline are still less soluble, therefore a great part of the substance remains in suspension. The diazotization of para-nitroaniline was carried out by adding at room temperature the sodium nitrite solution to a suspension of the finely powdered substance containing the required amount of hydrochloric acid. Small amounts of undissolved material were removed by filtration. This same method was applied to ortho-nitroaniline and 1, 2, 5-nitrotoluidine which were only used for the preparation of test antigens. In the case of the amino acids[4] as para-arsanilic acid, para-aminobenzoic acid, etc., the azoproteins can be dissolved without difficulty. For coupling diazotized ortho-aminobenzoic acid with horse serum, 50 cc. of normal NaOH per 100 cc. serum were used instead of sodium carbonate.

Immunization.—Eight rabbits were injected intraperitoneally with 15 cc. or less of each antigen at weekly intervals. At least two immune sera of sufficient strength were obtained after three to six injections in every case. Only one serum was obtained in the instance of para-chloroaniline.

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[4] For further details see Reference 3, pages 354–364.

Antigens for the Tests.—The azoproteins for the test solutions were prepared with chicken serum in place of horse serum in the manner described above. The diazo solution (in a few instances containing undissolved diazo compound) was added to a mixture of serum and sodium hydroxide (1 part of normal sodium hydroxide to 2 parts of serum) and the coupling allowed to take place at room temperature for half an hour. After precipitation with dilute hydrochloric acid the azoprotein was filtered and washed with water.

There was some difficulty in that the preparations did not yield clear solutions or were only sparingly soluble. This was overcome by treatment of the azoprotein with alkali. An amount of the antigen corresponding to 5 cc. serum was ground in a mortar, made up to 10 cc. with water and 10 cc. of normal NaOH was added. After half an hour the mixture was neutralized with 10 cc. normal HCl, the precipitated azoprotein centrifuged and taken up in about 25 cc. saline containing 0.5 cc. normal NaOH. After a few minutes hydrochloric acid was added until the solution was only weakly alkaline to litmus paper and the volume made up to 30 cc. The solution to which 0.5 per cent phenol had been added was clarified by intense centrifugalization and filtration through kieselguhr paper.[5]

To make the tests comparable also the antigens with acid azo components were treated with alkali in the same manner (concerning their preparation see Reference 3, pages 362 and 363). To obtain solutions of antigens made from the halogen substituted anilines it was necessary to prolong the treatment with alkali. A quantity of the azoprotein corresponding to 5 cc. serum was ground and 20 cc. of normal NaOH added. The mixture was shaken with beads for 2 hours, neutralized and the precipitated azoprotein was taken up in 25 cc. of saline containing 0.5 cc. normal NaOH. After addition of hydrochloric acid until the solution was weakly alkaline to litmus it was brought up to 30 cc., centrifuged and filtered through kieselguhr paper.

The quantity of antigen present in the solutions was determined by precipitating the azoprotein with alcohol, drying and weighing.

The solutions were found to contain from about 20 to 45 mg. of dry material in 5 cc.

Tests.—The antigens were diluted to 1:100 of a 1 per cent solution and 0.2 cc. of this solution was used for the tests. The tests were kept at room temperature and were in some instances also read after standing overnight in the ice box. The intensity of the reactions is indicated as follows: 0, f.tr. (faint trace), tr. (trace), ±, +, +±, etc.

Tests with Azoproteins Made from Aniline and Various Substituted Anilines.

Tables I and II illustrate the action of various immune sera on the azoproteins prepared from aniline and a number of substituted anilines.

[5] Macherey, Nagel and Co., Düren, Germany.

TABLE I.

Of the immune sera 779 and 788 four drops were used, and of the others 2 drops. The readings were made after 1 hour at room temperature.[6]

Azoproteins prepared from chicken serum and:	Immune sera obtained from antigens made from horse serum and:									
	Aniline	Aniline	Ortho-chloroaniline	Ortho-chloroaniline	Para-toluidine	Para-toluidine	Para-toluidine	Para-nitroaniline	Para-nitroaniline	Para-chloroaniline
	No. 760	No. 761	No. 788	No. 793	No. 763	No. 766	No. 770	No. 775	No. 795	No. 779
Aniline	++±	+±	+±	++	+±	+±	++	+	±	+
o-Toluidine	++	±	++	++±	+±	+	+	±	f.tr.	±
o-Anisidine	+	0	+±	++	±	tr.	0	0	0	0
o-Nitroaniline	+	0	+	+	tr.	tr.	0	±	tr.	0
o-Chloroaniline	+±	±	++	++±	+	+	+	±	+	tr.
m-Toluidine	++	+	+±	++	+±	+	+±	+	+	+
m-Nitroaniline	+	tr.	+	+	+	±	+	+±	+±	±
m-Chloroaniline	++	+	+	+±	+±	+	+±	+	+	+
m-Bromoaniline	++	+	+	++	+±	+	+	+	+	+
p-Toluidine	+±	+	+	+	++	+±	++±	+	+	++
p-Anisidine	++	±	±	+	++±	+±	++±	+	+±	+±
p-Nitroaniline	+	±	±	tr.	+±	+	+±	++	++	++
p-Chloroaniline	++±	+	±	+	++	+±	++±	+±	++	++
p-Bromoaniline	++	+	+	+±	++	++	++±	+±	++	++
p-Iodoaniline	+±	+	+	+	++	+±	++±	+±	+±	++

TABLE II.

1,2,4-Nitrotoluidine	++	±	+	++	+±	+±	++	+±	+±	+
1,4,2-Nitrotoluidine	++	±	+±	+±	++	+	+±	+±	+±	+
1,2,5-Nitrotoluidine	+	tr.	±	tr.	+	tr.	±	+±	+±	±
1,3,4-Xylidine	+±	±	+±	++	++	+±	++	+	+	+±
1,4,5-Xylidine	+±	tr.	+±	++	+	+	+	+	+	tr.
1,4,2-Xylidine	+±	tr.	+±	++	+	+	+	+	±	tr.
Acetyl-para-phenylene-diamine	0	0	0	0	0	0	0	0	0	0
Para-aminoacetophenone	tr.	0	0	0	+	tr.	±	+	±	0
Monomethyl-para-phenylenediamine	+±	+				+±	++	+±		

[6] The sera and the antigens are indicated by the names of the substances used in their preparation.

The experiments summarized in Table I show that the immune sera precipitate nearly all antigens. Also in most cases of negative reactions distinct precipitation occurred after standing overnight in the ice box and the weak reactions increased considerably in strength. Nevertheless a certain degree of specificity is to be observed.

The homologous reaction is always one of the strongest. The nature of the substituent is generally of but little influence; in the tests with

TABLE III.

4 drops were used of immune serum 729 and 2 drops of the others. The readings were made after 1½ hours at room temperature.

Immune sera obtained with antigens made from horse serum and:	Numbers of the immune sera	Azoproteins made from chicken serum and:										
		Para-aminobenzoic acid	Meta-aminobenzoic acid	Ortho-aminobenzoic acid	Para-arsanilic acid	Sulfanilic acid	Ortho-cinnamic acid	Aniline	Para-nitroaniline	Ortho-nitroaniline	Para-toluidine	Meta-toluidine
Para-amino-benzoic acid..	816	++±	±	0	0	0	0	0	0	0	0	0
Para-amino-benzoic acid..	818	+±	0	0	0	0	0	0	0	0	0	f.tr.
Ortho-amino-benzoic acid..	729	0	0	++	0	0	0	±	±	±		±
Para-arsanilic acid.....	722	0	0	0	++++	0	0	0	0	0		f.tr.
Aniline.....	760	0	0	0	0	0	0	++±	+	+	+±	++
Para-nitro-aniline.....	775	0	0	0	0	0	0	+	+±	+±	+	+
Para-toluidine..	770	0	0	0	0	0	0	++	+±	0	++±	+±

several immune sera however the intensity of the reactions was more diminished by the presence in the azo component of a nitro or a methoxy group than by the other substituents tested.

More pronounced is the effect produced by the position of the substituents regardless of their nature. In the tests with immune sera against para substituted azoproteins the strength of the reactions decreases in a general way in the order para, meta, ortho. This sequence is reversed in the reactions with sera against the ortho sub-

stituted aniline. Observations similar to these have been made previously (3, 5). The aniline immune sera seem to react somewhat more weakly with ortho substituted antigens than with the others.

No definite regularities appear from the tests of Table II except for the negative or weak reactions with the antigens prepared from acetyl-para-phenylenediamine and para-aminoacetophenone. The other antigens behave similarly to those included in Table I.

Tests with Azoproteins Prepared from Amino Acids.

A considerable number of immune sera prepared with acid azoproteins were described previously (3).

TABLE IV.

2 drops of aniline immune serum No. 760 were used. Readings after 1 hour.

Azoproteins made from chicken serum and the following substances:	Antigen dilutions 1 to			
	100	300	1000	3000
Aniline	+++	++±	+	±
Ortho-nitroaniline	+	+	tr.	0
Meta-nitroaniline	++	+±	±	tr.
Para-nitroaniline	+±	+±	±	tr.
Ortho-anisidine	+	+	±	tr.
Para-anisidine	+±	+±	±	f.tr.
Para-aminobenzoic acid	tr.	0	0	0
Sulfanilic acid	tr.	0	0	0

The present experiments as recorded in Table III demonstrate the considerable influence of acid groups on the specificity.

Table IV gives the results of tests made with various concentrations of the antigens.

The sera prepared with "non-acid" antigens give no precipitation with the "acid" azoproteins.[7] In the converse experiment showing the action of the "acid" immune sera on the "non-acid" antigens, there were weak positive reactions especially with one of the four sera and

[7] Distinct precipitation occurs in higher concentration of the acid antigens. These reactions were shown to be non-specific flocculations since such reactions took place also with entirely unrelated immune sera as precipitins for rat, pig serum, etc.

some more weak reactions came up after keeping the tests overnight. The reactions with serum No. 729 increased somewhat in strength.

A representative experiment is illustrated by Figs. 1 and 2.

In conformity with the findings already referred to (3) the sera for acid antigens exhibit in general a considerably higher specificity than the others. This is further substantiated by a series of tests in which the antigens for the reaction *in vitro* were made with the same protein as that used for the immunization (horse serum). Also in this way the difference between the two sorts of antigens is brought out clearly (Table V; *cf.* Fig. 3) although the method would tend to destroy the specificity of the reactions, due to the fact that the immune sera react to a certain degree upon the protein part of the antigens.

TABLE V.

To 0.2 cc. of the diluted antigen 1 drop of immune serum was added. The first reading was taken after 5, the second after 15 minutes.

Immune sera obtained with antigens made from horse serum and:	Numbers of the immune sera	Antigens made from horse serum and:										Horse serum	
		Aniline		Para-toluidine		Para-nitroaniline		Para-arsanilic acid		Para-amino-benzoic acid			
Aniline.....	760	++±	+++	+±	++	++	+++	tr.	±	0	tr.	±	+
Para-toluidine..	763	++	++	++	++±	++	++	tr.	tr.	0	0	+	+
Para-nitro-aniline....	735	+±	++±	+	++	++	+++	tr.	±	0	±	tr.	±

Reactions with an Azoprotein Made from the Methyl Ester of Para-Aminobenzoic Acid.

In order to prove in a different way the results obtained the following experiments were carried out. Starting from the ester of an aromatic amino acid an azoprotein was prepared. Presumably this should react with the non-acid immune sera like the other non-acid antigens. If this azoprotein is treated in such a way as to bring about hydrolysis of the ester it should no longer react with such sera but should have acquired by virtue of the free carboxyl group the property of reacting with an immune serum specific for the corresponding acid.

The methyl ester of para-aminobenzoic acid was prepared according to the directions given by Einhorn (6). The recrystallized substance melted at 112°C.

Diazotization.—0.190 gm. of the finely powdered substance was suspended in 10 cc. of water. Normal hydrochloric acid and a 2 per cent solution of sodium nitrite were added alternately in small quantities keeping the solution distinctly acid to Congo red and adding the nitrite after a test with potassium starch iodide paper showed the disappearance of nitrous acid. The theoretical quantity of nitrite was used up. The solution was kept at 0–5°C. This diazo solution was added to a mixture of 10 cc. of chicken serum and about 7 cc. of normal sodium carbonate, cooled to 0–5°C. The mixture was distinctly alkaline to phenolphthalein but an excess of alkali was avoided to prevent hydrolysis of the ester. After coupling for 10 minutes at 0–5°C., and acidifying with hydrochloric the azoprotein was filtered and washed with water. It was made up with saline into a fine suspension (volume 20 cc.) and made slighly alkaline to litmus by addition of 0.1 cc. of normal sodium hydroxide. Undissolved material was removed by centrifuging. The azoprotein was precipitated by neutralization with hydrochloric acid and after centrifugalization it was redissolved in 10 cc. of saline with alkali, carefully avoiding an excess. The solution which was very faintly alkaline to litmus was centrifuged and filtered through kieselguhr paper. Its content of azoprotein was estimated in the manner described before. The test solution was made up to 1/100th of a 1 per cent solution.

The experiment turned out as was anticipated (Table VI). The solution of the ester-azoprotein was precipitated by aniline and para-toluidine immune sera and not by an immune serum for para-aminobenzoic acid except for a weak reaction observed when the tests were kept overnight in the ice box. This is in agreement with the behavior of other non-acid azoproteins.

Hydrolysis of the Ester-Azoprotein.—To 2 cc. of a 1 per cent solution of ester-azoprotein were added 2 cc. of 1/10 normal sodium hydroxide and samples of 0.5 cc. were taken from the mixture at various times, *i.e.*, immediately after mixing, and after being kept 5 minutes, 1, 4, 6, 8 and 20 hours, at room temperature. These samples were adjusted to faint alkalinity with 1/10 normal hydrochloric acid, the azoprotein thus being kept in solution.

Table VII gives the reactions with the two immune sera at various stages of the hydrolysis. The antigen concentration corresponded in all cases to 1/100 of a 1 per cent solution.

TABLE VI.

2 drops of immune serum were used. Readings after 1 hour and after standing overnight in the ice box.

Azoproteins made from chicken serum and:	Immune sera obtained from antigens made from horse serum and:							
	Aniline		Para-toluidine		Para-nitroaniline		Para-aminobenzoic acid	
	No. 760		No. 770		No. 775		No. 816	
	1 hr.	Night	1 hr.	Night	1 hr.	Night	1 hr.	Night
Aniline	++	+++±	++	+++±	+	++	0	tr.
Para-toluidine	+±	++	++	+++±	+	++	0	tr.
Para-nitroaniline	+±	++	+±	++	+±	+++	0	±
Para-aminobenzoic acid	0	0	0	0	0	0	++	++++
Para-aminobenzoic ester	+	++	+	++	±	++	0	±

TABLE VII.

2 drops of immune serum were used. Readings after 1 hour at room temperature and after standing overnight in the ice box.

Azoproteins prepared with chicken serum:	Immune sera obtained with antigens prepared from horse serum and:			
	Para-aminobenzoic acid		Para-toluidine	
	No. 816		No. 770	
	1 hr.	Night	1 hr.	Night
Para-aminobenzoic ester azoprotein	0	tr.	+±	++
Same immediately after mixing with alkali	f.tr.	±	+±	++
Same after 5 min. hydrolysis	±	+	+±	++
Same after 1 hr. hydrolysis	+±	+±	+	+±
Same after 4 hrs. hydrolysis	+±	++	tr.	+
Same after 6 hrs. hydrolysis	++	++±	0	+
Same after 8 hrs. hydrolysis	++	+++	0	tr.
Same after 20 hrs. hydrolysis	++	+++±	0	tr.
Para-aminobenzoic acid azoprotein	++	+++±	0	tr.
Para-toluidine azoprotein	0	tr.	++	++±
Aniline azoprotein	0	f.tr.	++	++±

The experiment shows that during the course of the hydrolysis the precipitation with the serum for para-toluidine decreases gradually while correspondingly an increase in the strength of the reactions takes

place with the immune sera for para-aminobenzoic acid. The former reaction did not disappear completely but a trace of precipitate was still noticed after 20 hours hydrolysis when the tests were read on the following day.

SUMMARY.

The method of partial synthesis of antigens as employed in the foregoing experiments obviously cannot be substituted for the chemical study of natural antigens. But some questions of a rather general nature not easily accessible to investigations of the latter sort, may be approached by the use of artificial protein compounds. Thus the results reported indicate a peculiarity of certain chemical structures such as acid radicals.

The group of immune sera obtained by injecting azoproteins made from non-acid azo components had a wide range of activity. Substituents like CH_3, OCH_3, NO_2, Cl, Br, I, in the aromatic nucleus altered the reactions to a moderate degree only, in most cases.[8] The effects were dependent more on the position than on the nature of the substituents. Two substances were found, however, which had a pronounced effect on the specificity of the compound protein, namely acetyl-para-phenylenediamine and para-aminoacetophenone. In consideration of the above facts it is uncertain whether the antigenic changes noticed by Obermayer and Pick (7) after treating proteins with nitric acid, nitrous acid or iodine are mainly due to the substitution of hydrogen in the benzene ring by NO_2 and I, as is the general belief, or to other changes of the protein. This question had been raised already by the observation that the proteins treated with HNO_3 or HNO_2 containing respectively the nitro or the diazo group, did not differ substantially in their serological properties (8).

The antigens made from acid compounds form a group with distinctive features. In the first place the presence of acid radicals destroys the reactivity with the immune sera for the non-acid substances. This influence is so marked that even the reaction with the species-specific part of the protein, if such is present, appears to be diminished. Also the sera produced with the acid antigens react but feebly with the non-acid azoproteins. Accordingly it was pos-

[8] *Cf.* Wells,[1] page 79.

sible to show that by hydrolysis of the ester of an aromatic acid contained in an azoprotein the serological reactions of the antigen underwent a radical change.

The presence of a free carboxyl group in the antigens not only determines the characteristics mentioned but there is reason to believe that it increases markedly the degree of specificity exhibited by the antigens and the corresponding immune sera, when cross-tests are made with a number of acid azoproteins and their antisera. This is brought out by a comparison of the results of the present investigation with those described previously (3). It is of interest in this respect that the specific carbohydrates found by Avery and Heidelberger in pneumococci and pneumobacilli are mostly, if not in all cases, compounds of distinctly acid character.

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EXPLANATION OF PLATE 31.

FIG. 1. Reactions of immune serum for para-toluidine No. 770 with various azoproteins prepared from chicken serum and the following substances, readings after 1 hour:

1. Aniline.
2. Para-toluidine.
3. Ortho-chloroaniline.
4. Ortho-nitroaniline.
5. Meta-nitroaniline.
6. Para-nitroaniline.
7. 1, 3, 4-Xylidine.
8. 1, 2, 4-Nitrotoluidine.
9. Para-arsanilic acid.
10. Para-aminobenzoic acid.
11. Sulfanilic acid.
12. Saline control.

FIG. 2. Reactions of immune serum for para-aminobenzoic acid No. 816 with various azoproteins prepared from chicken serum and the following substances, readings after 1½ hours:

1. Aniline.
2. Para-toluidine.
3. Ortho-nitroaniline.
4. Para-nitroaniline.

5. Ortho-anisidine.
6. Para-arsanilic acid.
7. Ortho-aminobenzoic acid.
8. Meta-aminobenzoic acid.
9. Para-aminobenzoic acid.
10. Sulfanilic acid.
11. Ortho-aminocinnamic acid.
12. Saline control.

FIG. 3. Reactions of immune serum for aniline No. 760 with various azoproteins and with unchanged horse serum, reading after 15 minutes.

1. Azoprotein from chicken serum and aniline.
2. Azoprotein from horse serum and aniline.
3. Azoprotein from horse serum and para-toluidine.
4. Azoprotein from horse serum and para-nitroaniline.
5. Azoprotein from horse serum and para-aminobenzoic acid.
6. Azoprotein from horse serum and para-arsanilic acid.
7. Unchanged horse serum.
8. Saline control.

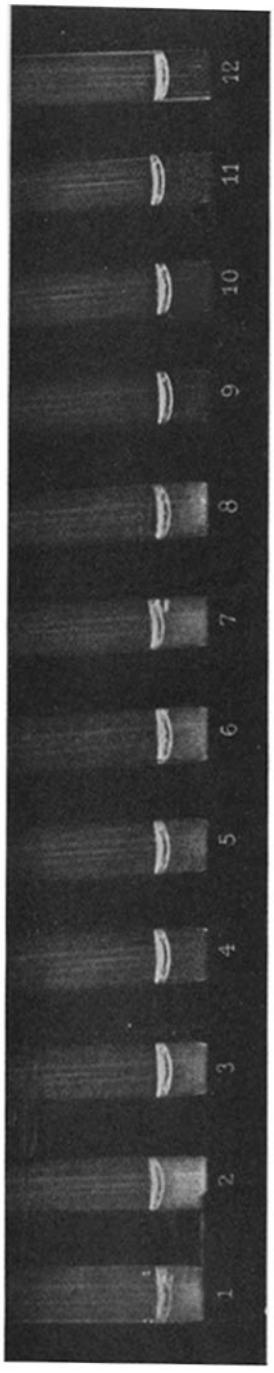

FIG. 1.

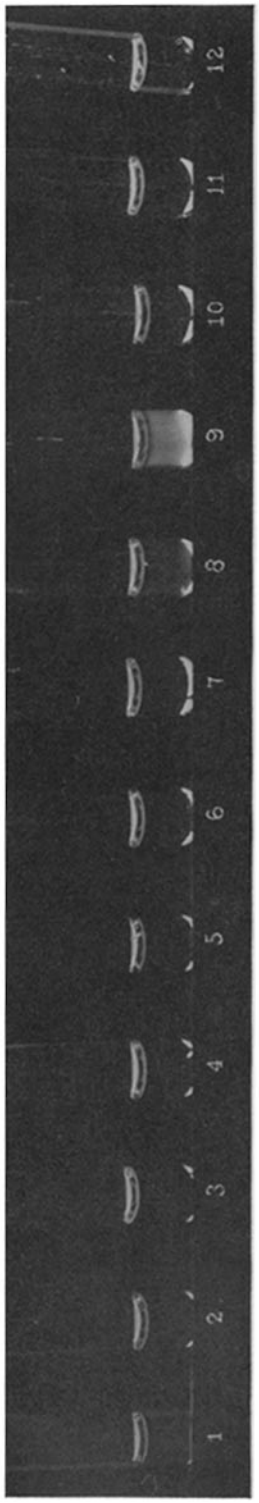

FIG. 2.

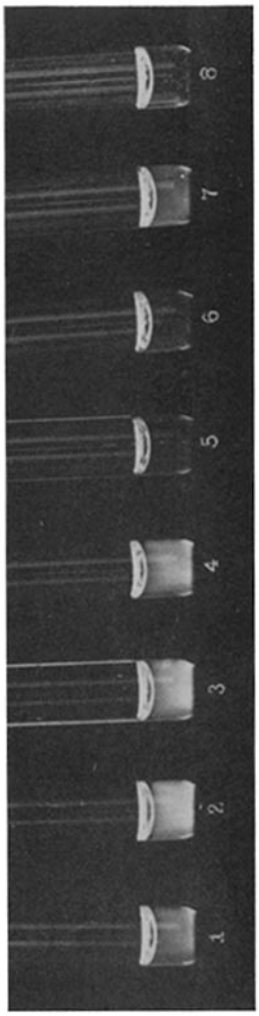

FIG. 3.

(Landsteiner and van der Scheer: Serological specificity of azoproteins.)