STUDIES ON THE PRECIPITIN REACTION

PRECIPITATING HAPTENS; SPECIES DIFFERENCES IN ANTIBODIES*

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The term "hapten" was applied by Landsteiner (1) to the portion of a complex antigen which determines its specific reactivity rather than the ability to function as an antigen. Thus in a complex azo protein the component attached to the protein through the azo group determines the specificity, while the protein molecule itself enables the complex to function as an antigen in the production of antibodies (2). Landsteiner demonstrated the specificity of the azo component (hapten) by its ability, when present in excess, to inhibit the specific precipitation of the antigen-antibody complex, and considered the inhibiting action to be due to actual combination of the hapten with the antibody.

It is now clear that the specific polysaccharides constitute a distinctive type of hapten which still retains the power to precipitate antibody specifically (3), combining chemically with the antibody in the precipitate and in the inhibition zone (4), just as Marrack and Smith (5) have recently found in the case of the inhibition reaction due to an azo compound.

It was originally assumed that the ability of specific polysaccharides to precipitate homologous antibodies was a function of a relatively high molecular weight (6). However, the writers have shown that the formula weights of the above mentioned specific carbohydrates are probably less than 10,000 (7). In the present communication there is described a series of precipitating haptens ranging from 550 to 1,800 in

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formula weight. These substances, partial hydrolysis products of the specific polysaccharide of Type III pneumococcus, precipitate Type III antipneumococcus horse serum and parallel in formula weight the series of hapten dyes recently described by Landsteiner and van der Scheer (8) as precipitating rabbit antisera.

A marked qualitative difference between antibodies to Type III pneumococcus engendered by the horse and those produced by the rabbit is brought to light by the failure of the partial hydrolysis products of S III[1] to precipitate the antibody in rabbit serum. Other differences of a quantitative nature will be discussed in a subsequent communication.

EXPERIMENTAL

Hydrolysis of S III with 70 per cent sulfuric acid and the separation of the hydrolysis products into fractions of different average molecular weight have been described in previous papers (9, 7). Unless the conditions are rigorously controlled, very stable sulfuric acid esters of the hydrolysis products may be formed. To eliminate this difficulty an additional series of fractions was prepared using hydrochloric acid as the hydrolytic agent.

The S III was suspended in 1:1 hydrochloric acid and hydrochloric acid gas was passed into the mixture until a clear solution resulted, keeping the temperature below 0°. The use of 1:1 acid as the initial solvent resulted in an even suspension of the S III and avoided the jelly-like lumps that formed when concentrated acid was used. The solution was allowed to stand in the ice box overnight. To guard against further hydrolysis of the higher fractions during the removal of the acid, they were precipitated by adding five volumes of 95 per cent alcohol and washed free from hydrochloric acid with alcohol before conversion into the barium salts. The supernatant and washings containing the lower fractions were concentrated to dryness *in vacuo*, keeping the temperature below 35°C. The residue was taken up in water, neutralized with barium hydroxide, and the barium salts were precipitated with alcohol and the process was repeated until the sugar salts were free from chlorides.

Any unhydrolyzed S III still present was removed as the copper salt by adding an excess of 5 per cent copper sulfate solution to a solution of the barium salts of the fractions, keeping the reaction neutral to litmus with barium hydroxide. After standing overnight the precipitate was centrifuged off and washed with water containing a little copper sulfate. The supernatant and washings were acidified with sulfuric acid and the copper was precipitated with hydrogen sulfide.

[1] This abbreviation is used to designate the specific polysaccharide of Type III pneumococcus.

After removing the excess of this gas by distillation *in vacuo*, the sulfuric acid was removed and the hydrolyzed S III converted into the barium salts by neutralizing with barium hydroxide. A portion of the solution was tested with copper chloride for traces of S III and the copper precipitation was repeated if necessary. It is possible that precipitation with copper salts removes, besides unchanged S III, higher hydrolytic products than those described in the present paper. Since it was necessary to remove all traces of S III the copper-precipitable material was discarded.

The barium salts were fractionally precipitated with alcohol after concentration of the solution *in vacuo* to a volume at which the barium salts began to separate. Addition of a little water yielded a clear solution. As alcohol was added, only slight precipitation occurred until a concentration of about 10 per cent of alcohol was reached, when considerable material was thrown down. This was centrifuged off and more alcohol was added. Again there was very little precipitation until at a concentration of about 25 per cent of alcohol a second large fraction was precipitated. After this had been removed the remaining barium salts were rendered insoluble by addition of five volumes of alcohol.

The behavior upon the addition of alcohol indicated that each fraction was not entirely homogeneous with regard to molecular weight but consisted of fragments of S III hydrolyzed approximately to the same extent and contaminated with relatively small amounts of material of higher and lower molecular weight. In the sulfuric acid series fractions B, C, and D were precipitated by 10, 25, and 80 per cent of alcohol respectively. In the hydrochloric acid series, in which the hydrolysis was less severe and the lower hydrolysis products were not formed, the corresponding fractions C and D have a larger average molecular weight. The precipitated fractions were washed with alcohol, filtered off, and dried *in vacuo* at 60°.

The aldobionic acid of S III (9) was prepared by allowing a solution of S III in concentrated hydrochloric acid to stand at room temperature for 2 days. After concentration *in vacuo* the barium aldobionate was isolated and converted into the brucine salt, which crystallized from a concentrated aqueous solution. After recrystallization it was reconverted into the barium salt.

The reducing power of the fractions was determined by the Willstätter-Schudel (10) and the Shaffer-Hartmann (11) methods, and the average molecular weight calculated from the mean of the two determinations, assuming one reducing group per molecule. A summary of the properties of the fractions is given in Table I. It must again be emphasized that each fraction is probably not a definite chemical individual, but represents a mixture of substances with not widely different molecular weights, the value given in each case being the average of the mixture. Fraction D in the sulfuric acid series corresponds roughly to a dialdobionic acid.

Solutions of the sodium salts of the fractions for use in the precipitin tests were made by treating solutions of the barium salts with a slight excess of sodium sulfate. The precipitin tests recorded in Table II were carried out with 0.5 cc.

portions of the dilutions of the fractions and 0.5 cc. portions of an antibody solution prepared from Type III antipneumococcus horse serum. The antibody solution contained 5.0 mg. of specifically precipitable protein per cc. Readings were taken immediately after mixing and after 2 hours in the water bath at 37° and overnight in the ice box. Corresponding tests with Type III antipneumococcus rabbit serum or antibody solution were negative, even after centrifugation.

DISCUSSION

It was originally reported that the partial hydrolysis products of S III did not precipitate Type III antipneumococcus serum (9). It has now been found that, even at a dilution of 1:1,000,000, all of the fractions except the aldobionic acid precipitate the homologous horse antiserum. The reaction differs from that of the unhydrolyzed S III in that the precipitating zone is narrower, chiefly owing to the inhibiting action of higher concentrations, as shown in Table II. Inhibition of precipitation, as read immediately after mixing, results in the concentrations used for testing in the previous studies, while under the normal conditions for the precipitin test a wider precipitating zone is found.

The possibility that the precipitate obtained is due to traces of unhydrolyzed S III in the fractions is excluded by several considerations. In the first place, unhydrolyzed S III is completely precipitated, even from very dilute solutions, by neutral copper sulfate. The supernatant from a copper-treated 1:20,000 dilution of S III contains less than 1:10,000,000 S III as shown by comparative precipitin tests. The purified hydrolytic fractions are not precipitated by copper salts under these conditions and even in high concentration do not inhibit the copper precipitation of added S III.

In the second place, quantitative determinations show that the amount of antibody precipitable by the fractions is much greater than could be caused by contamination with unhydrolyzed S III. The last two columns in Table I show the amounts of nitrogen precipitated from an antibody solution by 0.05 and 0.15 mg. of the different fractions and by S III for comparison. It will be seen that the hydrolysis fractions of higher molecular weight precipitate practically as much antibody as does an equal weight of S III. Moreover, a quantitative study of the entire range of precipitation[2] shows that the reaction in the case of the fractions differs from that of S III with antibody,

[2] To be reported in another communication.

TABLE I

Properties of Partial Hydrolysis Products of S III

Fraction	$[\alpha]_D$	Barium	Reduction calculated as glucose		Average formula weight	Precipitability by copper ion or rabbit antiserum	N pptd. by 0.05 mg. from 1 cc. antibody solution*	N pptd. by 0.15 mg. from 1 cc. antibody solution*
			Shaffer-Hartmann method	Willstätter-Schudel method				
	degrees	*per cent*	*per cent*	*per cent*			*mg.*	*mg.*
H_2SO_4 B	−27.2	17.2	9.8	10.3	1,800	−	1.21	1.68
H_2SO_4 C	−20.3	17.8	17.1	18.1	1,020	−	1.15	1.30
H_2SO_4 D	−7.2	17.2	32.5		550	−	0.31	
HCl C	−27.0	16.2	12.8	12.5	1,430	−	1.16	1.62
HCl D	−21.9	16.4	15.9	15.0	1,165	−	1.09	1.36
Aldobionic	+10.6	18.8	50.0		360	−	−	−
S III	−34.0				5,600†	++++	1.23	2.12

* Prepared from antipneumococcus horse serum by Felton's procedure (13). Analyses according to (14).

† See Reference 7.

TABLE II

Precipitin Reaction between Hydrolysis Products of S III and Antipneumococcus Horse Serum, Type III

Fractions in order of decreasing molecular weights	Dilution of fraction								
	1:250	1:500	1:1,333	1:2,000	1:4,000	1:40,000	1:400,000	1:1,000,000	1:5,000,000
S III	(−)	(±)	(+)	(++)	(+++)	(++++)	(+++)	(±)	(−)
	±	++	++++	++++	++++	+++±	+++	++	+
H_2SO_4 B	(−)	(−)	(−)	(±)	(++)	(++)	(+)	(±)	(−)
	−	−	+±	++±	+++	+++±	+++	++	+
HCl C	(−)	(−)	(−)	(±)	(++)	(++)	(+)	(±)	(−)
	−	−	+±	++	+++	+++	+++	++	+
HCl D	(−)	(−)	(−)	(−)	(+)	(++)	(+)	(±)	(−)
	−	−	−	+±	++	+++	+++	++±	+
H_2SO_4 C	(−)	(−)	(−)	(−)	(+)	(+++)	(+)	(−)	(−)
	−	−	+	+±	++	++±	++	++	−
H_2SO_4 D	(−)	(−)	(−)	(−)	(−)	(++)	(+)	(−)	(−)
	−	−	−	±	+±	++±	++	+	−
Aldobionic	−	−	−	−	−	−	−	−	−

Parentheses indicate readings taken immediately after mixing. Other readings after 2 hours at 37°, overnight in ice box.

chiefly in the greater inhibiting effect of increasing concentrations. This is also shown qualitatively in Table II.

Finally, rabbit antisera that precipitate heavily with unhydrolyzed S III are not precipitated by any dilution of the fractions, even when the concentration of protein specifically precipitated by S III is as high as 17 mg. per cc. of serum. Combination of the fractions with rabbit antibody occurs, nevertheless, to form soluble compounds, since relatively high concentrations of the fractions inhibit specific precipitation with S III. Felton has shown that antibodies to Pneumococcus in horse sera are precipitated with the water-insoluble fraction of the serum globulins (12). Rabbit antipneumococcus sera, however, yield no precipitate on dilution, so that it is possible that the failure of the hapten fractions to form insoluble compounds with rabbit antibody may be connected with the greater tendency of rabbit globulin to remain in solution. This tendency, however, does not prevent even as dilute a solution of S III as 1:10,000,000 from precipitating rabbit antibody.

The difference between rabbit and horse antibodies to Pneumococcus as regards their ability to precipitate the S III fractions raises the question whether or not other haptens of low molecular weight might more often prove to be precipitating as well as inhibiting were immune horse sera used instead of the rabbit sera now almost universally employed. Thus Landsteiner and van der Scheer have observed innumerable inhibition reactions with simple haptens, but reported positive precipitin tests only in the case of the azo dyes formed by coupling *p*-aminosuccinanilic acid and its homologs with resorcinol or tyrosine (8).

SUMMARY

1. Partial hydrolysis products of the specific polysaccharide of Type III pneumococcus ranging from 550 to 1,800 in formula weight can be quantitatively freed from unhydrolyzed polysaccharide.

2. The fractions yield specific precipitates with Type III antipneumococcus horse serum but fail to precipitate homologous rabbit antisera, giving rise only to specific inhibition. The aldobionic acid, the structural unit of S III, does not precipitate antisera.

3. A possible explanation and a possible application of the findings are pointed out.

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