IMMUNOLOGICAL REACTIONS BETWEEN AGAR-AGAR AND SOME BACTERIAL ANTISERA

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Heidelberger, Goebel and Avery (1) found an immunological relationship between Type II pneumococcus and Type B Friedlander bacillus, and accounted for this relationship by a probable chemical similarity between the specific carbohydrates of the two organisms. Zozaya (2) found a similar reaction between the polysaccharides of meningococcus, *B. anthracis*, *B. proteus*, *B. subtilis* and *B. mesentericus*. Zozaya (3) found a similar immunological relation between dextran polysaccharide and some bacterial antisera. He attributed these reactions also to a probable chemical similarity between the carbohydrates of these substances.

Sordelli and Mayer (4) have recently reported that sera prepared by immunizing animals with agar cultures of *B. anthracis* and *E. typhi* contain precipitins for agar. These findings suggested our present studies, the more so since they confirmed the belief of Furth and Landsteiner (5) and Heidelberger (6) that specific polysaccharides prepared from organisms grown on agar would contain agar as an impurity, a fact necessitating a partial revision of our previous work (3).

Methods

The agar used in the experiments was the reagent agar Merck. It gave no reducing sugars before hydrolysis and 38 per cent after hydrolysis, contained 2.7 per cent ash and 0.27 per cent nitrogen. The biuret, ninhydrin, xanthroproteic and Millon reactions were negative. Positive reactions were obtained by the Tollens and the orcinol tests for pentoses, confirming Fellers' (7) findings. The agar also gave a strong starch-iodine test.

Adsorption.—The organisms used, Pneumococcus Type I and *Streptococcus viridans* (Bargen), were grown in hormone broth for 24 hours at 37°C. and centrifuged. No preservative was added. The mass of organisms was thoroughly

mixed with 0.1 per cent agar and left in the ice box overnight. They were then washed five times with salt solution, centrifuging each time. After the last washing the organisms were suspended in salt solution and standardized by turbidity to 2,000 million organisms per cc.

Immunization of Animals.—Rabbits were injected three times a week intravenously with increasing doses (0.5 to 2 cc.). After 6 weeks they were bled. As controls, rabbits were similarly injected with agar in a dilution of 1:10,000 and others with a dilution of 1:2,000,000. Rabbits were also injected with Pneumococcus Type I and *Streptococcus viridans* (Bargen) agar-free, as controls.

Serum from a horse immunized with meningococcus grown on agar (59843) which had a high titer to agar, was also used.

Tests.—The precipitin tests were made by mixing equal amounts of the polysaccharide and serum dilutions and incubating for 3 hours at 37°C. The agar precipitate is usually flaky, somewhat transparent and difficult to read. To overcome this difficulty the tubes were centrifuged before reading the test.

The bacterial polysaccharides here used were prepared from the organisms grown in bouillon, with the exception of the meningococcus polysaccharide 40, which was prepared from organisms grown in agar. The C substance (8) was prepared from a pneumococcus R Type II strain obtained through the kindness of Dr. O. T. Avery of The Rockefeller Institute.

EXPERIMENTAL

Table I shows the precipitin test of the antimeningococcus serum (59843) with agar and with the meningococcus polysaccharide. This test shows the very high reactivity of the agar-agar. Negative results were obtained with 40 normal horse sera. Normal rabbit serum also gave negative reactions.

Table II shows the result of the agar precipitin test made with sera of rabbits immunized with Pneumococcus Type I and *Streptococcus viridans* (Bargen) after absorption with agar and with agar adsorbed on collodion particles, as well as with sera of control rabbits injected with agar alone in two different dilutions. It is of interest to note the lower titer serum obtained with the agar adsorbed on collodion particles as compared with the titer of the pneumococcus and streptococcus. This difference may well be due to differences in the susceptibility of the animals. The agar alone did not produce antibodies detectable even in very low dilutions of the serum.

Precipitation with the homologous polysaccharide was not influenced by adsorption of agar on the organisms used for producing these antisera. Tables III and III, *a*, show precipitin tests with the sera of rab-

bits immunized with agar adsorbed on Pneumococcus Type I, absorbed and non-absorbed with the Pneumococcus Type I polysaccharide, and with agar, demonstrating the independence of the two antibodies. These tables also show the results of the precipitin reaction of the same serum absorbed and non-absorbed with C substance of the pneumo-

TABLE I

Precipitin Test with Antimeningococcus Serum (59843) and Meningococcus and Agar Polysaccharides

Dilution of polysaccharide	1:8,000	1:16,000	1:32,000	1:64,000	1:2,000,000
Meningococcus polysaccharide 40	3	2	2	1	0
Agar	4	4	4	4	1

Incubated for 3 hours at 37°C.

For reading this and the following tables:

1 = slight suspended precipitate.
2 = slight sedimenting precipitate.
3 = moderate sedimenting precipitate.
4 = marked sedimenting precipitate.

TABLE II

Agar Precipitin Test with Sera of Rabbits Immunized Respectively with Pneumococcus Type I, Streptococcus viridans (Bargen), Collodion Particles Adsorbed with Agar and Agar Alone

Animal injected with	Agar dilution				
	1:50,000	1:100,000	1:500,000	1:1,000,000	1:2,000,000
Pneumococcus Type I	4	3	2	2	1
Streptococcus viridans	3	4	1	0	0
Collodion particles		3	1	0	0
Agar 1:10,000	0	0	0	0	0
Agar 1:2,000,000	0	0	0	0	0

Incubated for 2 hours at 37°C. and overnight in the ice box.

coccus, with meningococcus polysaccharide 40 and with agar. Here we notice a relationship of the C substance, meningococcus polysaccharide and agar which will be discussed later.

Table III, *b*, shows the results of the precipitin tests with the sera of rabbits immunized with agar-free Pneumococcus Type I, with C

substance, agar and meningococcus polysaccharide (No. 40). It is plainly shown that this serum contains some antibodies to the C substance but not to agar or to the meningococcus polysaccharide.

TABLE III

Homologous Polysaccharide Precipitin Tests with the Sera of Rabbits Immunized with Pneumococcus Type I and with Streptococcus viridans (Bargen) with and without Adsorption with Agar

Animal injected with	Polysaccharide dilution					
	1:20,000	1:40,000	1:80,000	1:160,000	1:500,000	1:1,000,000
Pneumococcus Type I adsorbed with agar	4	3	3	2	2	1
Pneumococcus Type I agar-free	4	4	3	2	1	1
Streptococcus viridans (Bargen) adsorbed agar	4	2	0	0	0	0
Streptococcus viridans (Bargen) agar-free	4	3	1	1	0	0

Incubated for 3 hours at 37°C. overnight in ice box, and centrifuged before reading.

TABLE III, *a*

Precipitin Tests with Pneumococcus Type I SSS, C Substance of the Pneumococcus, Meningococcus Polysaccharide (No. 40) and Agar, on Absorbed and Non-Absorbed Serum with the Same Substances, of a Rabbit Immunized with Agar Adsorbed on Pneumococci Type I

(Serum dilution 1:4)

Serum absorbed with	Control non-absorbed	Agar	Pneumococcus Type I SSS	Meningococcus (40)	C substance
Agar (1:10,000)	2	0	3	1	0
Pneumococcus Type I SSS (1:1,000)	4	4	0	4	4
Meningococcus (1:1,000)	3	0	3	1	0
C substance (1:1,000)	3	1	2	0	0

Incubated 3 hours at 37°C.—overnight in ice box—centrifuged.

Organisms grown on agar definitely adsorb some of the agar. To show this we studied the agglutination of organisms grown in bouillon, on agar and on agar after being washed five times with saline, with a serum (antimeningococcus) which had no agar antibodies and a similar

serum which had. The results are shown in Table IV. The bouillon cultures gave no reaction with either of the two sera, with the exception

TABLE III, *b*

Precipitin Tests with Serum of Rabbit Immunized with Pneumococcus Type I, with the C Substance of Pneumococcus, Agar and Meningococcus Polysaccharide (No. 40) Respectively

Dilutions of polysaccharide	1:4
C (1:1,000)	2
	2
Agar (1:10,000)	0
	0
No. 40 (1:1,000)	0
	0

Incubated 3 hours at 37°—overnight in ice box—centrifuged.

TABLE IV

Agglutination Tests Made with Organisms Grown in Bouillon and Agar with Anti-meningococcus Serum. (A without Antibodies and B with Agar Antibodies)

Organism	*Staphylococcus albus*						*B. typhosus*					
Grown on	Bouillon		Agar		Agar washed 5 times		Bouillon		Agar		Agar washed 5 times	
Serum used	A	B	A	B	A	B	A	B	A	B	A	B
1:4	0	2	0	4	0	2	0	0	0	4	0	2
1:8	0	2	0	4	0	1	0	0	0	4	0	1
1:16	0	0	0	4	0	0	0	0	0	4	0	0
Organism	Pneumococcus Type I						*B. proteus* × 19					
Grown on	Bouillon		Agar		Agar washed 5 times		Bouillon		Agar		Agar washed 5 times	
Serum used	A	B	A	B	A	B	A	B	A	B	A	B
1:4	0	0	0	4	0	2	4	4	4	4	4	4
1:8	0	0	0	4	0	1	4	4	4	4	4	4
1:16	0	0	0	3	0	0	3	3	3	4	3	3

Incubated 3 hours in water bath at 37°C.

of *B. proteus* × 19, which shows the expected cross-reaction (3). The same organisms grown on agar all showed a marked agglutination with

the serum containing agar antibodies and not with the other. Finally the organisms grown on agar and then washed five times with saline gave very slight reactions with the serum containing agar antibodies but not with the control serum. This suggests that even after five washings there yet remains sufficient agar adsorbed to give a positive agglutination reaction.

To study further the relation of this agar antibody to the specific bacterial polysaccharide antibody, a polysaccharide was prepared from meningococcus grown in broth and free from agar. To this polysaccharide we have given the designation of A-. The anti-meningococcus serum which contained agar antibodies was absorbed

TABLE V

Titration of Serum 59843–2 after Absorption with No. 40, A- and Agar 1:1,000 with Same Polyssacharides (1:1,000)

Serum dilution	Absorbed No. 40			Absorbed A-			Absorbed agar			Control		
	No. 40	A-	Agar	No. 40	A-	Agar	No. 40	A-	Agar	No. 40	A-	Agar
1:2.....	2	4	0	4	0	4	3	4	0	4	4	4
1:4.....	1	3	0	3	0	4	3	3	0	4	4	4
1:8.....	1	2	0	2	0	3	2	2	0	3	3	3
1:16....	1	0	0	1	0	2	1–	1	0	2	2	2–

Incubated in water bath 3 hours at 37°C.

separately with polysaccharides A-, No. 40 (which contained agar) and agar in a dilution of 1:1,000, then titrated against each of the above carbohydrates, using the non-absorbed serum as control. The results of this test are shown in Table V. Again the independence of the homologous and agar antibodies is shown.

Having observed that the C substance of the pneumococci crossed with the antimeningococcic serum, reacted up to a dilution of 30:50,-000 while the meningococcus polysaccharide reacted at 500,000 with the same serum, we were led to investigate the relation between agar, the C substance and the meningococcus polysaccharide. We absorbed a polyvalent antipneumococcus serum (Types I and II) with C substance, meningococcus polysaccharide 40 and with agar, all in a dilution of 1:1,000. With the absorbed sera we made precipitin

reactions with the three polysaccharides, using as control the non-absorbed sera. The results are shown in Table VI.

We notice that agar removed part of the meningococcus carbohydrate antibodies and part of C substance antibodies and all of the agar antibodies. Polysaccharide 40 absorbed a great part of the C substance and agar antibodies, while C substance absorbed its homolo-

TABLE VI

Precipitin Tests Made with Meningococcus Polysaccharide (No. 40), C Substance of the Pneumococcus, and Agar-Agar and an Antimeningococcus Serum Containing Anti-Agar Antibodies and a Pneumococcus Polyvalent Serum, Absorbed with Same Polysaccharides and Non-Absorbed

Serum.....	Meningococcus control			Meningococcus absorbed with agar			Meningococcus absorbed with No. 40 (2)			Meningococcus absorbed with C (2)		
Dilution.....	1:4			1:4			1:4			1:4		
Polysaccharide...	No. 40	C	Agar	No. 40	C	Agar	No. 40	C	Agar	No. 40	C	Agar
Non-diluted..	4	4	4	2	2	0	2	1	0	3	1	0
1:2.....	4	4	4	2	2	0	2	1	1	2	0	0
1:4.....	4	4	4	1	2	0	1	1	2	2	0	0
1:8.....	4	4	4	1	1	0	0	1	2	0	0	0

Serum.....	Pneumococcus control		Pneumococcus absorbed with agar		Pneumococcus absorbed with No. 40 (2)		Pneumococcus absorbed with C (2)	
Dilution.....	1:4		1:4			1:4		
Polysaccharide...	C	Agar	C	Agar	C	Agar	C	Agar
Non-diluted..	4	1	4	0	4	0	0	0
1:2.....	4	1	4	0	3	0	0	0
1:4.....	4	2	4	0	3	0	0	0
1:8.....	4	2	4	0	3	0	0	0

Incubated 3 hours at 37° and overnight in ice box.

gous antibodies and also agar antibodies and a small part of the meningococcus polysaccharide antibodies.

In the case of the pneumococcus serum, agar absorbed its homologous antibodies as we have shown before, but did not absorb any of the C substance antibodies, while the C substance absorbed the agar and its homologous antibodies completely. These results show that the C substance contains the active group or groups of the agar molecule.

DISCUSSION

The fact that agar-agar when adsorbed on bacteria or other colloids is antigenic becomes of great importance in the practical consideration of non-specific cross-reactions in agglutination or precipitin tests in which the organism or the precipitin antigen is prepared from cultures grown on agar. It is true that antibacterial sera, when prepared with antigens grown on agar, are not usually employed sufficiently long in immunization for the animal to produce an anti-agar antibody and the probable error is much reduced. But in animals which are immunized for long periods (6 weeks or more) the danger becomes evident.

The work of one of us (3) in which a relationship was shown between the polysaccharides of meningococcus, *B. anthracis*, *B. proteus*, etc. is now found to be quantitatively defective as all of the polysaccharides used were prepared from organisms grown on agar and so were contaminated with it in the final product. However, repetition of the work with carbohydrates prepared from bouillon cultures continues to give the same cross-reactions although in a lesser degree than previously reported.

The cross-reactions observed with the C substance of the pneumococcus and agar, as well as the meningococcus polysaccharide and agar, suggest that the meningococcus polysaccharide we used is more of the C type, *i.e.* common to all the Gram-negative cocci, as reported by one of us (9). It is difficult to know if the specificity remaining in the meningococcus polysaccharide is of the nature of the true type specificity which Rake (10) has found in recently isolated cultures. We have not yet found such a specific substance.

Regarding the C substance of the pneumococcus, Tillett and Francis (11) have shown some reactions with this substance in the serum of patients suffering with streptococcus and staphylococcus infections and suggest a certain relationship to Gram-positive cocci.

The relation of agar, C substance and meningococcus polysaccharide to other bacterial antisera is being studied, notably those of *E. typhi*, *B. anthracis*, some of the Salmonella group and others.

CONCLUSIONS

1. As found by Sordelli and Mayer, agar adsorbed on bacteria produces agar-reacting antibodies in animals injected with these organisms.

2. False cross-agglutination and precipitin reactions can be produced in sera containing agar-reacting antibodies by organisms grown on agar. Zozaya's publication in this field (3) requires partial revision on this account.

3. There is suggestive immunological evidence of chemical similarity between the specifically reactive groups of agar-agar, and the C substance of the pneumococcus.

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