THE ANTIBODY-FORMATION BY POLYSACCHARIDS

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That antibodies are formed for lipoids had long been doubted. Recently, however, this doubt has been cleared away by the production of the antibodies (1) (2) in animals injected with lipoids and serum proteins.

It has occurred to me that polysaccharids, which belong to the same sort of emulsoids, might be endowed with antigenic properties and that their action as antigens might be facilitated by using serum proteins or other substances as vehicle.

The fact that glycolytic ferment develops in the animal body after the injection of sugar has been demonstrated by Weinland, Abderhalden, and others. There is, however, little literature dealing with the antigenic nature of sugar. Heidelberger and Avery (3) (4) have isolated a substance which bore close resemblance to polysaccharids from the pneumococci. This substance precipitated antipneumococcic serum, but immunization experiments with it have given a negative result. Rokuro Kondo (5) obtained an antilipoidal serum by the use of a mixture of lipoids with inulin.

I have experimented with inulin, soluble starch and dextrine, and succeeded in obtaining antisera against all of them. The results were reported in the December issue of the *Japan Medical World*, Home Edition in 1927 (6), and in the *Saikingaku Zasshi* (*Journal of Bacteriology*) April, 1928 (7). I shall deal with the same matter in this report, reviewing all I have reported previously in addition to what I have done since.

Methods

The three kinds of polysaccharids used in my experiments were of Merck make. I prepared solutions of them and injected them into the postauricular veins of rabbits daily for some time. On the 5th to 6th day from the last injection the blood of the animals was collected, and the serum was separated and inactivated

at 56°C. for ½ hour. The test was made by complement fixation and precipitation, using the corresponding polysaccharids as antigens.

Complement Fixation Test.—The serum was put in a series of 6 test tubes containing 0.2, 0.1, 0.05, 0.025, 0.0125 and 0.006 cc. of it respectively, made to 0.5 cc. with saline. Tube 1 was a control, containing serum only. To the remaining 5 tubes 0.5 cc. of the antigen was added. The complement consisted of 0.5 cc. of diluted guinea-pig serum, containing 2.5 lytic units. The mixtures were kept for 1 hour in the incubator, and then 1 cc. of the hemolytic system was added (0.5 cc. of a 5% goat erythrocytal suspension and 0.5 cc. of the anti-sheep serum having 3 times as strong a hemolytic power) incubation done for 2 hours more and the results were read.

The inulin, used as antigen in the complement fixation test was a 2% solution, the soluble starch a 1.5% solution, and the dextrine a 3% solution in saline. All were dissolved by heating. Each of these antigen-solutions (1.5 cc.) was tested and found to give no auto-inhibition.

EXPERIMENTAL

A. Inulin

Material for Injection.—Inulin was dissolved in saline to the proportion of 4% and heated at 70°C. Prior to injection, 0.8 cc. of the solution was mixed either with 0.2 cc. of the pig serum or with the same amount of the saline. The mixture was then put in the incubator for 1 hour. The controls received pig serum only, in the same dilution.

Doses and Number of Injections.—Each of the three mixtures described above was injected in a dose of 1 cc., 1.5 cc., 2 cc., 3 cc., 4 cc., 5 cc., and 6 cc., each dose being repeated.

Complement Fixation Test.—The normal serum often gave a very weak positive reaction. These positive sera, however, gave a negative reaction when they were inactivated by heating at 60°C. for 1 hour. They also gave a negative reaction when the mixture was allowed to stand at 0°C. for 1½ hours instead of at 37°C. before the hemolytic system was added. From these facts it will be seen that the positive reaction, which the normal sera gave, was non-specific in nature.

Results of complement fixation tests with the sera of injected rabbits are given in Table I.

From Table I, it will be seen that the sera of the rabbits, which had been treated either with inulin solution alone or with inulin-pig-serum mixture, all gave positive results. The control serum from the rabbit injected with the pig serum alone, gave a negative reaction.

Precipitation Tests.—These were carried out by both the mixing and the ring precipitation methods, but both yielded negative results.

Bunji Imai (8) has reported that by the complement fixation test he has demonstrated the antigenic power of inulin. He also obtained immune sera, which gave precipitation with a certain kind of inulin.

B. Soluble Starch

All the immunization experiments with a weak starch solution proved unsuccessful, but by the use of a strong one, the antibody formation was at last induced.

TABLE I

Results of the Complement Fixation Test with the Sera of the Rabbits Receiving Inulin Only

Rabbit No.	Serum (cc.)					
	0.2	0.1	0.05	0.025	0.0125	0.006
1	L	H	H	H	H	K
2	L	H	H	H	H	k
3	L	H	H	K	L	L
4	L	H	K	L	L	L
5	L	H	K	L	L	L
6	L	H	K	L	L	L

Results of the Complement Fixation Test with the Sera of the Rabbits Receiving Inulin Plus Pig Serum

7	K	H	H	K	k	L
8	k	H	H	H	K	k
9	L	H	H	H	K	L
10	H	H	H	H	K	L
11	H	H	H	H	H	K

H stands for inhibition, K or k incomplete hemolysis and L complete hemolysis in the complement fixation.

Material for Injection.—A 3% soluble starch saline mixture was heated and made into a pasty substance. It was used without any vehicle.

Doses and Number of Injections.—A daily injection with a dose of 0.5 cc. was given for 18 days.

Complement Fixation Test.—There was no case in which normal serum gave a positive result. After the completion of one course of injections, the sera of all the treated animals gave positive results, as shown in Table II.

Precipitation Test.—The tests all gave negative results.

Ken Nodzu (9) as well as B. Imai (8) have demonstrated the antigenic power of soluble starch by the complement fixation test. Nodzu

TABLE II

Results of the Complement Fixation Test with the Serum of the Rabbits Receiving Soluble Starch

Rabbit No.	Serum (cc.)					
	0.2	0.1	0.05	0.025	0.0125	0.006
12	L	H	H	K	L	L
13	L	K	L	L	L	L
14	L	H	K	L	L	L
15	L	H	H	H	K	L
16	L	H	K	L	L	L

TABLE III

Results of the Complement Fixation Test with the Serum of the Rabbits Receiving Dextrine Only

Rabbit No.	Serum (cc.)					
	0.2	0.1	0.05	0.025	0.012	0.006
17	L	K	L	L	L	L
18	L	K	L	L	L	L
19	L	K	k	L	L	L
20	L	K	k	L	L	L
21	L	L	L	L	L	L
22	L	L	L	L	L	L

Results of the Complement Fixation Test with the Serum of the Rabbits Receiving Dextrine Plus Pig Serum

23	K	H	H	H	H	K
24	K	H	K	L	L	L
25	K	H	K	L	L	L
26	L	H	H	H	K	L
27	K	H	H	H	H	K
28	L	H	H	H	K	L
29	K	H	H	H	H	K
30	L	H	H	K	L	L

stated that he, like myself, obtained only negative precipitation tests, but Imai states that he obtained a weakly positive reaction.

C. Dextrine

Experiment 1

Material.—The dextrine used in my experiments was a white powder, which gave a purplish indigo color with iodine. It was insoluble except when heated; and therefore must have been amylodextrine. (For convenience sake it is stated as "D.I.") A 6% mixture of D.I. with the saline was made by heating at 70°C. The pig serum was added in the same proportion as in the case of inulin.

Dose and Number of Injections.—These were the same as in the case of inulin.

Complement Fixation Tests.—The normal sera gave positive reactions less frequently than in the case of inulin, and gave less marked reactions. By differential testings, the reaction was proved to be nonspecific.

The results of the complement fixation tests with the sera of rabbits treated with dextrine alone or with the mixture of dextrine and pig serum are shown in Table III. It will be seen that the serum of the rabbits immunized with the mixture of dextrine and pig serum gave a strong reaction, whereas those from animals receiving dextrine alone yielded doubtful reactions. The control serum from the rabbit injected with pig serum alone, gave a negative reaction.

Precipitation Reaction.—The results were entirely negative. Thus far, dextrine had induced antibodies only when injected in association with pig-serum. Because of the fact, however, that inulin and soluble starch gave antibodies even though injected without the addition of pig-serum, I next submitted animals to an increased amount and number of injections, in order to determine whether or not the addition of pig-serum is absolutely necessary to effect the antibody formation.

Experiment 2

Material.—The kind of dextrine and the concentration of its solution were the same as employed in Experiment 1.

Dose and Number of Injections.—18 injections with a dose of 5 cc. were given.

Complement Fixation Tests.—The results of the complement fixation tests made with the sera of the treated animals are shown in Table IV. It will be seen that dextrine when given by itself elicited antibodies.

Experiment 3

Material.—I used two different kinds of Merck dextrine, both being further decomposed than that of the previous experiments. One was a white powder given a purple color with iodine, and easily soluble. (For convenience sake this

lot is called "D.II.") The other was a yellow granular substance giving a red color with iodine and also easily soluble. (This is called "D.III.") D.III. was found to be erythrodextrine. In degree of decomposition, D.II. stood just between D.I. and D.III. The D.II. and D.III. specimens were dissolved in saline to a proportion of 6%, and injected into animals without the addition of the pig serum.

Amount and Number of Injections.—18 injections were given with a dose of 5 cc.

Complement Fixation Test.—All the sera gave negative results.

Experiment 4

Material.—A 6%, 10% and 15% saline solution of the D.II. and D.III. were prepared.

Amount and Number of Injections.—One course of treatment consisted of 5 injections each of 6%, 10% and 15% solutions in saline, the dose being 10 cc. The total amount of the injected material was, therefore, about three times as much as it was in the previous experiments.

TABLE IV

Results of the Complement Fixation Test with the Serum of the Rabbits Receiving Dextrine Only

Rabbit No.	Serum (cc.)					
	0.2	0.1	0.05	0.025	0.012	0.006
31	L	H	H	K	L	L
32	L	H	K	L	L	L
33	L	H	H	L	L	L
34	L	H	H	K	L	L

Complement Fixation Test.—The results of the complement fixation test with the sera of the animals treated with D.II. and D.III. were all negative. It may be inferred that these two kinds of dextrine were lacking in antigenic action.

From the results of my experimental immunization with dextrine, it might be concluded that the results of Imai, as stated above, who could not successfully immunize the animal with seven repeated injections of 5 cc. of a 6% dextrine solution as such or mixed with 0.5 cc. of the inactivated pig serum, were due either to an insufficient number of injections or the use of an unsuitable kind of dextrines.

Specificity of the Antibody

After heating the immunized sera at 60°C. for 1 hour, no changes in the complement fixation reaction occurred. Nor was the reaction

influenced by carrying out the first half of the procedure of the test at 0°C. for ½ hour. From these facts, it may be concluded that the reaction is of a specific nature.

In order to determine the specificity of the immune serum reaction against the above mentioned three polysaccharids, I carried out cross tests with them. In this experiment, I used a 1.5% solution of the three polysaccharids as antigen. The results are shown in Table V. Each gave a specific reaction.

TABLE V

Results of the Complement Fixation Tests for the Specificity of the Immune Bodies Derived from Polysaccharids

Antigen	Soluble starch				Inulin				Dextrine (D.I.)			
Amount of sera cc	0.1	0.05	0.025	0.012	0.1	0.05	0.025	0.012	0.1	0.05	0.025	0.012
Soluble starch immune rabbit serum:												
No. 15	H	H	H	K	L	L	L	L	L	L	L	L
No. 12	H	H	K	L	L	L	L	L	L	L	L	L
No. 16	H	K	L	L	L	L	L	L	L	L	L	L
Inulin immune rabbit serum:												
No. 11	L	L	L	L	H	H	H	K	L	L	L	L
No. 10	L	L	L	L	H	H	K	L	L	L	L	L
No. 5	L	L	L	L	H	K	L	L	L	L	L	L
Dextrine (D.I.) immune rabbit serum:												
No. 29	L	L	L	L	L	L	L	L	H	H	H	K
No. 30	L	L	L	L	L	L	L	L	H	H	K	L
No. 31	L	L	L	L	L	L	L	L	H	K	L	L

It may be noted here that the D.I. immune serum did not give any positive reaction to D.II. or D.III.

Very recently, Ken Nodzu (10) immunized an animal with starches from Indian corn, barley, wheat, potato, etc., and Yoshio Masuda (11) with those from two varieties of rice. Each of the immune sera reacted most strongly against that kind of starch which had been used as the antigen, while against others the reaction was very weak. The authors, therefore, insisted that they could immunologically differentiate starches, which it is very difficult to do by morphological features.

Fate of the Antibodies in the Animal Body

Blood was collected from the immunized rabbits at the end of every week and the complement fixation test was carried out with sera heated at 60°C. for 1 hour. The results are shown in Tables VI, VII and VIII.

TABLE VI

Results of the Complement Fixation Test with the Sera of Rabbits into Which Inulin Was Injected

Day of collection of blood	Results of the complement fixation with		
	Serum of Rabbit 10	Serum of Rabbit 11	Serum of Rabbit 5
1st week	H H H K L L	H H H H H K	L H K L L L
2nd week	L K k L L L	L H H H K L	L K L L L L
3rd week	L K L L L L	L H H K L L	L L L L L L
4th week	L L L L L L	L H H L L L	
5th week		L H L L L L	
6th week		L L L L L L	

TABLE VII

Results of the Complement Fixation Test with the Sera of Rabbits into Which Soluble Starch Was Injected

Day of collection of blood	Results of the complement fixation with		
	Serum of Rabbit 12	Serum of Rabbit 13	Serum of Rabbit 14
1st week	L H H K L L	L K L L L L	L H K L L L
2nd week	L H K k L L	L L L L L L	L K L L L L
3rd week	L K L L L L		
4th week	L L L L L L		

TABLE VIII

Results of the Complement Fixation Test with the Sera of Rabbits into Which Dextrine Was Injected

Day of collection of blood	Results of the complement fixation with		
	Serum of Rabbit 29	Serum of Rabbit 30	Serum of Rabbit 28
1st week	K H H H H K	L H H K L L	L H H H K L
2nd week	L H K L L L	L K K L L L	L H K L L L
3rd week	L K L L L L	L L L L L L	L H K L L L
4th week		L L L L L L	L K L L L L
5th week			L L L L L L

It will be seen that the sera in the period following the immunization lost their strength rapidly, those of animals weakly immunized giving a negative result in a fortnight, while the strongly immunized gave one in 6 weeks.

Do the Polysaccharids Themselves Elicit the Formation of Antibodies?

The above mentioned polysaccharids gave rise to antibodies, even when unassociated with vehicle. But is it the polysaccharids as such that cause the antibodies to develop, or may a trace of proteins co-mingled in the materials play the part of vehicle? To settle the matter I have carried out an experimental investigation.

In the first place, I made Millon's and biuret tests upon the three kinds of polysaccharid solutions. They all gave a negative result. I then tried quantitative estimation of the nitrogen in the specimens after Kjeldahl's microestimation method. The results were that inulin contained 0.036%, soluble starch 0.017% and dextrine (D.I.) 0.019% of nitrogen.

Are the Nitrogen Contents Proteins?

In order to find whether the nitrogenous substances are proteins the following experiments were carried out:

To 200 cc. of a 2% hydrochloric acid solution 20 gm. of soluble starch was added, and the mixture was hydrolyzed by being boiled for 3 hours. The liquid was then neutralyzed and subjected to the iodine reaction test. It proved negative. It was, therefore, certain that all the starch had been converted into glucose. For the purpose of removing the salts and glucose, some of the liquid was then put into a bladder and left in running water for 48 hours. Then, the remaining liquid in the bladder was evaporated to 1/10 volume. The concentrate had a blackish brown color, one so dark that it was impossible to make color reaction tests with it. It was found, however, to contain 0.01% of nitrogen on quantitative estimation. The liquid gave also a positive reaction of Heller's ring test.

Another portion of the hydrolyzed solution, which had been neutralized, was allowed to evaporate to 1/10 without being dialyzed and a quantitative estimation was made of its nitrogen contents. It was found to contain 0.011% of nitrogen. This liquid also gave a positive result with Heller's ring test.

From the results of the above described experiments, it seems certain that the nitrogen found in the soluble starch solutions was protein.

Is the nitrogen, which is found in inulin or dextrine, of protein origin?

This theme remains to be studied. It will probably be hard to free these polysaccharids perfectly from nitrogenous substances.

From all the accessible data it might be inferred that it would be difficult to determine whether or not proteins play an important part as the vehicle in the immunization processes, but from the following two facts, proteins are suspected to be intimately related with the antibody production by polysaccharids.

1. Considering the connection between concentration, dose and number of injections of the used polysaccharid solutions and also the amount of the produced antibodies, it might be judged that inulin gives rise to antibodies comparatively easily. However, it is hard to obtain immunization, with soluble starch and dextrine. The nitrogen contents of the latter two is only about one half that of the first.

2. Comparing the results of the complement fixation tests with the immune sera of rabbits injected with inulin and dextrine with and without pig-serum, it will be seen that while there did not occur any differences with inulin, which is relatively rich in nitrogen, with dextrine, which is poor in nitrogen, the serum of the animal treated with the mixture of dextrine and pig-serum gave by far the stronger result. (See Tables III and IV.)

The facilities for successful immunization depend largely upon the natural characteristics of the materials injected and the nature of the animals, yet from the above mentioned two facts it may well be thought that there is an intimate connection between the production of immune bodies for polysaccharids and the association with them of proteins.

The possibility arises that the antibodies demonstrated by the complement fixation test, might have been directed against incidental protein. This is rendered rather unlikely by the following considerations.

1. As described above, the dialyzate obtained from the hydrolyzation products of the starch solution yielded an absolutely negative iodine reaction, but was positive to Heller's test. However, the complement fixation test made with this dialyzate and the anti-starch serum proved negative.

2. The polysaccharid solutions, which were used as antigens, gave no color reaction of proteins. Their nitrogen contents was at a

minimum and probably was not protein. Granting all of it to have been such, the total amount of nitrogen found in the material employed as antigen in the complement fixation test was extraordinarily small. On calculation the proteins in 0.5 cc. of the polysaccharid solutions, which I employed in my test, would be about 8/1,000,000 gm. in the starch specimens and 20/1,000,000 gm. in those of inulin. The real protein contents was doubtless but a fraction of these calculated protein contents. It is unlikely that these traces should produce such a remarkable complement fixation reaction as I have obtained.

CONCLUSIONS

1. By complement fixation tests, it has been clearly demonstrated that the sera of rabbits immunized with inulin, soluble starch and dextrine contain specific antibodies.

2. All these immune sera gave a negative precipitation reaction.

3. The kind of dextrine which has a construction very near to starch has an antigenic property, but those in a state of further decomposition do not give rise to antibodies.

4. All the three kinds of polysaccharids have power to produce antibodies without any vehicle. Dextrine is the only one of the three that gives rise to immune bodies more readily when pig serum is added to it.

5. Regarded as antigens, inulin stood first and soluble starch and dextrine next in order.

6. All three kinds of polysaccharids that were employed gave a negative protein color reaction. All of them, however, contained nitrogen. It has been proved that the large portion of the nitrogen contained in the soluble starch is derived from its protein contents.

7. It is suggested that in the production of immune bodies by these three kinds of polysaccharids, proteins might play the part of the vehicle. This is, however, still to be determined.

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