STUDIES ON THE ANTIGENIC SUBSTANCE OF THE BACTERIAL CELL.

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In the course of a number of years during which we have occupied ourselves with the study of bacterial antigens, we have become convinced that the progress of immunological knowledge has been not inconsiderably impeded by the tendency to apply all the facts ascertained by the study of coagulable proteins, such as horse serum, egg white, etc., by direct analogy to the reactions induced in animals infected or treated with bacteria. Our earlier studies (1) on the differentiation between the bacterial allergies, such as the tuberculin reaction and protein anaphylaxis, persuaded us that we could not hope to make consistent progress in the further understanding of the biology of bacterial infection and immunity unless we could obtain more precise knowledge concerning the bacterial antigenic substances which, in quite obvious chemical properties, differed from the antigenic true proteins.

Our studies with Parker since that time (2), and those of a number of our associates, have occupied themselves with a variety of bacteria, including tubercle bacilli, pneumococci, meningococci, staphylococci, and typhoid, colon, and influenza bacilli, and our associate, Mueller (3), has carried out special studies with yeast and, with Smith and Litarczek, with bacilli of the Friedländer group. These investigations have yielded results that are, in certain fundamental aspects, consistent among themselves and, in large part, in harmony with the very important studies carried on by Avery and Heidelberger (4) along closely related problems.

The conditions may be summarized at the present time, briefly, as follows:

None of the bacteria studied so far contain any considerable amount of heat-coagulable albumin or globulin. These materials are probably always present to a slight extent in bacterial extracts and may determine the limited formation of antiprotein antibodies and, consequently, of true anaphylaxis. But they do not represent the predominant antigenic constituents of the bacterial cell.

Occasionally, small amounts of a substance acting like Bence-Jones protein has been found, especially in extracts of tubercle bacilli, but this, when present, is small in amount and of undetermined significance, though, as Bayne-Jones (5), Hektoen and Welker (6), and others have shown, this, too, may form its own homologous antibodies.

All the bacteria investigated have yielded considerable amounts of two substances which have seemed to be of particular importance, and which differ, both chemically and in their biological attributes, from the antigenic constituents of animal blood serum and perhaps of tissues.

One of these is the material which we have referred to in a number of papers as the "residue substance," and which we believe to be identical with the biuret-free, precipitable, but not antigenic, digests of typhoid extracts described by Pick as early as 1912 (7), surely the same as the "soluble" pneumococcus substance observed by Dochez and Avery (8) in the urine and blood of pneumonia patients and in the filtrates of broth cultures. This material may be obtained by a variety of methods from all bacteria so far investigated by us. It is diffused out into fluid culture media; it may be extracted from the ground or unground bacterial sediments, either by simple extraction or after moderate antiformin treatment; and from some bacteria, notably pneumococci, meningococci, and influenza bacilli, it may be easily washed off the bacterial surfaces by brief shaking in neutral isotonic solvents. It is relatively heat-stable; gives no protein reactions; is alcohol-precipitable; and, according to the precise chemical studies on pneumococcus made by Heidelberger and Avery (9), with which the investigations of Mueller on yeast and tubercle bacilli (10) agree, their structure is that of a complex carbohydrate.

Biologically these substances are precipitable, give complement fixation reactions with homologous antibodies, and are specific—so delicately group-specific, indeed, that there seems little doubt about their representing the particular material upon which this property of the whole bacteria depends. In the form in which they are obtained separate from the bacteria, however, they are completely incapable of inducing antibody formation. The ease of their removal in the case of capsulated organisms has quite obviously suggested to us, as it has to Avery, their possible morphological involvement with capsular material, a thought which is strengthened by the possibility that their capacity to unite with, *i.e.* divert, antibodies outside the living cellular structures may explain the insulating protective functions of the bacterial capsule.

The second material which has invariably been obtained from all bacterial extracts, and in quantities which have seemed to determine it as the bulk of the extractable bacterial substance, is of a protein nature, since it gives qualita-

tive protein reactions, but was designated by us in our first paper as "nucleoprotein," largely because it is precipitable by acids in the cold, at quite definite pH ranges, redissolving at neutrality and slight alkalinity, and in excess of acid. The exact chemical definition of this material must await the collection of sufficient amounts for systematic analysis—a task now being undertaken by Mueller and his associates in this laboratory, but it is quite obviously not an ordinary coagulable protein, since, if boiled in acid suspension while it is flaking out, these flakes immediately redissolve when the solvent is neutralized to pH 6.9 to 7.2.

This last material is also antigenic, not as strictly specific as the residue,[1] but capable under certain conditions not only of reacting *in vitro* with antibodies, but also of inducing antibody formation when injected into animals. As studied so far, the nucleoprotein is probably a mixture of a number of substances not functionally separable up to the present time. The various biological properties we attribute to it, therefore, must be regarded for the present as those of the impure substance which may, on further study, be again divisable into a number of functional parts. Thus, we have already described, as a result of the studies of Mueller and those of Mueller with one of us (11), that the nucleoprotein fraction of tuberculin and tubercle bacillus extracts seems to carry down with it in an impure condition the material responsible for the tuberculin reaction, and in this function is entirely separable from the residue. This is being further investigated at the present time by Mueller.

In our first study in 1921 on the tuberculin reaction, in which the separation of the bacterial substances into the two fractions noted above was carried out, we suggested the possibility that the nucleoprotein might represent "the mother substance from which the residue material was derived." Later, in studying the antibody reactions and confirming our earlier failures to obtain antibodies by injection of residue materials in spite of prolonged efforts, we mentioned the possibility that the residue material constituted what Landsteiner (12) had spoken of as "haptenes" and since that time the extreme specificity of these materials, together with their impotence to incite antibodies, has led us to speak of them as the "haptophore group" of the bacterial cell. We suggested at that time that molecular size and anti-

[1] We do not include experiments on the specificity of nucleoprotein antibody reactions in the text in the interests of brevity. Cross-reactions with nucleoproteins and antisera of streptococcus, pneumococcus, and tubercle bacilli showed group reactions with the concentrated material between streptococcus and pneumococcus nucleoproteins, union with either of them and the tubercle bacillus antiserum of group reactions, however, being eliminated in dilutions of 1:10. In connection with this see, also, the work of Lancefield.

body formation might be related, the residue material representing the haptophore group that had been split off from the larger molecule, union with which had made it a complete antigen. We tried at that time to obtain residue material by subjecting nucleoprotein to digestion in the autoclave at various hydrogen ion concentrations and various pressures, and last year attempted a similar experiment with horse serum and egg albumin, believing that we might be able to split off such a haptophore group from these materials, but also without success (13).

In the experiments presented in the present paper we have attempted to obtain a better understanding of the relationship between these antigenic fractions of the bacterial cell by immunizing a series of animals with whole bacteria and nucleoproteins obtained from tubercle bacilli, streptococci, and pneumococci and comparatively studying the antibodies obtained as a result.

As will be seen in the protocols in the very simple experimental procedures recounted below, the materials used throughout were whole bacteria, undissolved and dissolved, and nucleoproteins produced by the ordinary methods and employed both unfiltered and after filtration through Berkefeld filters for the removal of all bacterial fragments.

Experiment 1. Tubercle Bacilli.

February 4, 1925. 50 cc. of ten times concentrated synthetic bacillus broth precipitated with $\frac{N}{1}$ HCl. Centrifugalized. Precipitated nucleoprotein taken up in 50 cc. salt solution at pH 7±. Supernatant fluid precipitated by the fractional alcohol precipitation of Mueller. Two volumes of 95 per cent alcohol added. Centrifugalized. Precipitate discarded. Three further volumes (volume counted as original volume of material) now added to the supernatant fluid. Centrifugalized and redissolved in 50 cc. of salt solution. We now have three substances for test:

1. The original whole material.
2. The nucleoprotein precipitate from 50 cc. again dissolved in 50 cc. of salt solution and therefore representing probably somewhat less than the original concentration of this material in the whole broth.
3. The residue material precipitated by fractional alcohol precipitation redissolved in 50 cc. of salt solution and likewise, allowing for loss, representing somewhat less than the original concentration of this material in the whole broth.

Precipitations with this material were now done with two sera:

1. Serum A, obtained from a rabbit immunized with living H 37 tubercle bacilli.

2. Serum B, obtained from a rabbit immunized systematically with unfiltered nucleoprotein.

Results of precipitations were as follows:

	1			2		
	Serum A—living H 37 tubercle bacilli.			Serum B—nucleoprotein.		
Dilution.	Whole material.	Nucleo-protein.	Residue.	Whole material.	Nucleo-protein.	Residue.
Concentrated.	−	++ to +++	−	−	−	−
1:5	+++	+	+++	+++	+++	+++
1:10	+++	±	+++	+++	+++	+++
1:20	+++	−	+++	+++	+++	+++
1:40	+++	−	+++	+++	+++	+++
1:80	+++	−	+++	+++	+++	+++
1:100		−	++	++ to +++	++	++
1:200			+	+	+	+ to +++
1:300			+	−	±	−
1:400			±	−	±	−
1:500			−	−	−	−

It will be seen from this experiment that the antiserum produced with whole tubercle bacilli had little precipitating power for the nucleoprotein material; whereas that produced by the injection of nucleoprotein material precipitated both the residue and the nucleoprotein.

This relationship could not, however, be accepted as a conclusive definition of the true facts, since the nucleoprotein material with which Rabbit B (2) had been treated had not been filtered, and it was possible that the activity of its serum upon residue might have been due to the bacilli and bacillary fragments undoubtedly present in the nucleoprotein in spite of repeated resolution and reprecipitation. We therefore repeated this work with filtered nucleoprotein, made just as before from alkaline extracts of tubercle bacilli and passed through Berkefeld candles. In doing this work we have not been

able to explain why it has been so exceedingly difficult to induce any kind of antibody formation with the filtered nucleoprotein of the tubercle bacillus. This, however, is the case and it was only by persistent effort and the injection of large amounts of the filtered material that the results recorded in Experiment 2 were finally obtained.

This experiment shows that when the nucleoprotein is filtered free of any traces of bacterial fragments no antibody to the residue is formed.

Experiment 2. Tubercle Bacilli.

Comparison of Antibodies in Sera Prepared with Unfiltered and with Filtered Nucleoprotein Respectively versus Tubercle Bacillus Nucleoprotein and Residue.

	Serum C (nucleoprotein unfiltered).	Serum D (nucleoprotein filtered).	Serum E (nucleoprotein filtered).
Nucleoprotein.			
1:100			
1:250	++++	+++	+++
1:500	+++	++ to +++	++
1:1000	++	+	±
	+	±	±
Residue.			
Concentrated.	+++	±	−
1:5	+++	−	−
1:10	+++	−	−

With streptococci similar conditions appear to prevail, as is apparent from the following experiment which was performed incidental to streptococcus immunizations done with the Dick strains of scarlatinal origin, at this time, by one of the writers with Dr. F. B. Grinnell.

Experiment 3. Hemolytic Streptococci.

Rabbit F, immunized with whole streptococci sediment from broth cultures.
Rabbit G, immunized with streptococcus nucleoprotein.

Precipitations.

	Streptococcus Serum F (whole bacteria).	Streptococcus Serum G (nucleoprotein serum).
Nucleoprotein.		
Concentrated.	++++	++ slow.
1:5	+++	++
1:10	+++	+
1:50	+++	±
1:100	+	−
Residue.		
Concentrated.	++++	−
1:5	++	−
1:10	+	−
1:50	−	−

Here, again, it is apparent that while the whole bacteria induce antibodies both to the nucleoproteins and to residue, immunization with filtered nucleoproteins fails to induce any antibodies which react with the residue material.

It thus appears, from the foregoing, that when the bacterial body is extracted by the relatively gentle method of treatment with a weak alkali, and this extract then fractionated by the acid precipitation method, two substances are obtained—one the nucleoprotein which seems to be independently antigenic, inducing antibodies which react only with itself and not with the residue; the other the residue material incapable of inducing any kind of an antibody reaction, but capable of reacting with antibodies formed by injection of the whole bacteria. It would seem, from this, that the residue represented the haptophore group of something left behind in the bacterial bodies during the extraction. Since it is obviously quite without promise to approach closer to a solution of this problem by immunizing with the surely complex mixture of the sediment remaining after the extractions, we determined to try the following, using pneumococci largely because of the ease with which these organisms can be dissolved in bile:

1. To immunize a rabbit with intact, undissolved pneumococci.

2. To immunize another animal with all the materials obtained in pneumococcus solutions after filtration to remove all formed elements.

The two animals would thus, to all intents and purposes, receive the

total pneumococcus substances—in the one case, however, intact and morphologically complete—in the other, after solution.

It is, of course, impossible to devise methods of doing this which, on the one hand, can be held to avoid completely the injection of dissolved materials and, on the other, safeguard against the possibility of chemical change during solution. Conscious of this, however, we chose what we thought would represent the nearest approach to the desired conditions.

Experiment 4. Pneumococcus.

1. Pneumococci were grown on blood agar in pie plates. They were washed off with 2 per cent formalin solution, allowed to stand a few minutes, twice washed in salt solution, and immediately injected—intraperitoneally.

2. Similarly grown pneumococci were dissolved in the smallest amounts of ox bile which appeared to give complete solution, filtered through Berkefeld candles and similarly injected. A number of animals were lost in this process, probably because of the toxicity of the bile.

We did not drive these sera up very high, because the procedures were poorly tolerated by the animals and we did not wish to delay our work. Moreover, we believed that the basic principles could be demonstrated by relatively low titer sera.

	Serum H. Rabbit injected with formalinized pneumococci.	Serum I. Rabbit injected with bile-dissolved pneumococci.	New York State antipneumococcus serum.
Nucleoprotein.			
Concentrated.	+++	+++	
1:5	++	++	+++
1:10	++	++	+++
1:20	++	+	+++
1:50	++	±	++
1:100	+	±	++
1:200	+	−	+
1:400	−	−	±
Residue.			
Concentrated.	++	−	Not done.
1:5	++	−	" "
1:10	+	−	+++
1:20	+	−	++
1:50	−	−	+
1:100	−	−	−
1:200	−	−	−

SUMMARY.

Putting together the results of such experiments as those outlined above, we can set down the following definite facts.

1. The substance of the bacterial cell can be roughly divided into two antigenic entities. One of these is the so called "nucleoprotein" substance, the other the residue substance or soluble material of Dochez and Avery and Avery and Heidelberger, both of which have been repeatedly characterized in preceding papers.

2. Immunization with the nucleoprotein, if such nucleoprotein is rendered free of bacterial bodies or fragments of bacterial bodies by Berkefeld filtration, incites the production only of antinucleoprotein antibodies which, with slight group overlapping, are species-specific but, as determined by the previous studies of Avery and, subsequently, those of Lancefield, are not type-specific to the same degree as the residue antibodies.

3. Immunization with dissolved residue alone leads to no antibody formation whatever. This residue, as indicated in several of our own previous studies, represents the haptophore group upon which specificity depends and which, in the simple process of solution, is disrupted from another substance together with which it represented a complete antigen in the antibody-forming sense.

4. The formation of specific antiresidue antibodies is apparently dependent upon the injection of morphologically formed elements, at least as far as experiment can determine at the present time; for, as in the pneumococcus experiments, the most available process of solution and the injection of all the materials so obtained from the whole bacteria fails to yield antiresidue antibodies, as though in the mere process of dissolving the residue haptophore group were dissociated from its association with the larger molecule to which, in the whole bacteria, it lends specificity.

5. While antiresidue antibodies are only formed when such undisrupted bacterial cell substances are present in the immunizing substance, immunization with whole bacteria, even when attempts are made to preserve them from solution by formalin, leads to the formation of both antiresidue and antinucleoprotein antibodies, probably because a certain amount of solution inevitably takes place after injection within the animal body.

DISCUSSION.

In our earlier publications we assumed that the residue haptophore group is probably split off from the nucleoprotein, which may be regarded as its mother substance. Attempts to approach this by splitting residue off from nucleoprotein after it was obtained by the ordinary methods by digestion at various hydrogen ion concentrations, etc., were failures. As the matter stands at the present time, there are two possible interpretations. On the one hand, we may assume that the bacterial body contains two separate antigenic complexes—one concerned with the ectoplasmic capsule-forming zone in which the residue material, together with some protein substance, represents an antigenic union easily disrupted and separate from the nucleoprotein basic substance of the bacterial cell. This original residue-protein combination when split by solution would then yield two separate substances, neither of which is alone antigenic, and the residue recognizable later only by its ability to react with antibodies formed with material in which the two are still united and, therefore, antigenic. The only antigenic substance left in the dissolved material, then, would be the nucleoprotein, which would represent the second antigenic complex of the bacterial material constituting its bulk, and it is more cytoplasmic than specific in an entirely separate system from the one involving the residue. This would involve assuming that the second constituent of the original residue complex is entirely lost in experiments like the ones cited above.

A simpler explanation would be to return to our original interpretation, that the antigenic complex of the whole bacterial cell consists of a combination of nucleoprotein and residue material in which the residue determines the specificity of the total, just as in the work of Landsteiner certain methyl substances, etc., may alter the ecificity of proteins to which they are attached. We are at the present time inclined to favor the latter position, first of all because it is simpler, and in the second place because when we inject the total dissolved pneumococci we get only antinucleoprotein antibodies. Moreover, it seems to be the antiresidue antibodies which determine agglutination and perhaps their reactions toward whole bacteria and antiserum, a subject which is still under investigation.

The only absolutely crucial experiment that could finally determine this matter in a simple way, would be success in synthetically reuniting residue with nucleoprotein. Since there seems no immediate hope of attaining this, we believe that indirect methods such as the one we have indicated above must be persisted in for the time being.

BIBLIOGRAPHY.

1. Zinsser, H., *J. Exp. Med.*, 1921, xxxiv, 495.
2. Zinsser, H., and Parker, J. T., *J. Exp. Med.*, 1923, xxxvii, 275.
3. Mueller, J. H., and Tomcsik, J., *J. Exp. Med.*, 1924, xl, 343. Mueller, J. H., Smith, D. E., and Litarczek, S., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 373.
4. Avery, O. T., and Heidelberger, M., *J. Exp. Med.*, 1923, xxxviii, 81.
5. Bayne-Jones, S., and Wilson, D. W., *Bull. Johns Hopkins Hosp.*, 1922, xxxiii, 37.
6. Hektoen, L., and Welker, W. H., *J. Infect. Dis.*, 1924, xxxiv, 440.
7. Pick, E. P., in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganismen, Jena, 2nd edition, 1912, i, 781.
8. Dochez, A. R., and Avery, O. T., *J. Exp. Med.*, 1917, xxvi, 477.
9. Heidelberger, M., and Avery, O. T., *J. Exp. Med.*, 1923, xxxviii, 73; 1924, xl, 301.
10. Mueller, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 209.
11. Zinsser, H., and Mueller, J. H., *J. Exp. Med.*, 1925, xli, 159.
12. Landsteiner, K., *Biochem. Z.*, 1918–19, xciii, 106.
13. Zinsser, H., *J. Immunol.*, 1924, ix, 227.