STUDIES ON PATHOGENIC B. COLI FROM BOVINE SOURCES.

IV. A Biochemical Study of the Capsular Substance.

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The carbohydrate gums which have been obtained from bacteria in sufficient quantity for chemical study are few in number. The early workers were concerned with capsular material as such, and the possibility of its precipitation by homologous immune serum had not been thought of. A bibliography and review of the literature are to be found in the papers of Heidelberger and Avery (1), or of Heidelberger (2).

Preisz (3) studied in detail the rôle of capsule and capsular material in infectious disease. He made preparations of capsular material in considerable amount. He believed it to be a mucin and stated that it gave no reducing sugar reaction. Whether the substance was prepared from virulent or avirulent strains, it removed from sera the factors responsible for lysis or the killing of anthrax bacilli (page 399). But he did not consider this a specific reaction, because similar material from cultures of organisms resembling *B. anthracis* also removed bactericidal substances from antianthrax sera *in vitro*. He thought that in the last stages of infection in the rabbit, capsular substance was present in blood and exudate in solution, neutralizing the antibody. He maintained that there was throughout a parallelism between virulence and the possession of a capsule.

With observations from studies such as this two lines of evidence converged towards and merged into the present concept of a polysaccharide reacting specifically with homologous serum, responsible for type specificity, but not eliciting antibody formation, and in all probability connected with the possession of a capsule and virulence in certain cases.

1. In 1921 Zinsser (4) called attention to the immunological importance of the non-protein constituents of bacterial cells. This frac-

tion which reacts with specific serum antibodies was termed "residue" in order not to imply too accurate an idea of its chemical structure. He believed this to represent the so called "haptene" of Landsteiner (5), for Landsteiner in his studies upon chemical modifications of precipitable antigens foretold the probable existence of materials which would react with antibodies without themselves being capable of inducing antibody formation when injected into the animal body. These conditions Zinsser's "residue" fulfilled, and the suggestion was made that they represented the haptophore group split off from the antigen as a whole and molecularly too small to induce antibody formation. It was also stated that this "residue" was analogous to the substances previously found by Cole (6) and by Dochez and Avery (7) in the blood and urine of typhoid and pneumonia patients.

2. The mass of evidence presented in the fundamental papers of Avery and Heidelberger (2) and Heidelberger and Avery (2) leaves us without grounds for doubting that it is a protein-free preparation that reacts specifically with homologous precipitating antibody and is responsible for type specificity. Very marked chemical differences were found correlated with serological specificity.

In the work of Mueller (8) and of Heidelberger, Goebel, and Avery (9) it was suggested that the "residue" or soluble specific substance is either identical with or connected with the capsule. But a question very naturally arises, why then is it possible to obtain "residue" from such apparently uncapsulated organisms as the tubercle bacillus, the staphylococcus, the meningococcus, and the influenza bacillus (10), as well as from the capsulated types of pneumococcus and the Friedländer bacillus? Is the capsule due to an increased production of a substance always present in the bacterial cell, a substance capable of reacting with antibody, but not of eliciting its formation on injection? Such a substance might represent the "haptophore" group of Ehrlich, and meet the conditions of the "haptene" prophesied by Landsteiner. Another question that naturally arises is the relation between capsular substance and virulence. A brief survey of the literature at once shows us that the possession of a capsule is almost universally regarded as increasing the virulence of an organism. This might be brought about in two ways. In the first place the capsular substance might act as a morphological factor, not dissolving away from

the organism, and insulating it from antibody action. Secondly, it might protect the organism by reacting in solution, at some distance from the bacterial cell, with antibody.

EXPERIMENTAL.

The possession of a number of mutating cultures of *B. coli* described by Theobald Smith and associates (11–13) offered an opportunity to look for the answers to the above questions in the case of this one species. It should be possible to find out if more precipitable carbohydrate is obtainable from the capsulated than from the non-capsulated strain from equal areas of culture surface, what its probable relation to the greater virulence of the capsulated strain may be, as well as something about its chemical properties.

A consideration of the literature leads us to expect that a capsule would increase the virulence of an organism. This is, therefore, a very unique opportunity to obtain a quantitative estimate of the extent to which it enhances virulence. We think we are justified in excluding other factors, because the organisms are so alike. One, called the (b) form, is derived as a mutant from a colony of the other, called (a). Precipitation in the culture filtrate of either one is caused by the serum of the other, as well as by its own serum, as will be shown later. Filtrates of bouillon cultures, 24 to 48 hours old, of both (a) and (b) forms when injected into calves exert marked toxicity (11), but guinea pigs are only slightly sensitive.

These cultures of *B. coli* also afford an opportunity to test out the suggestion that "residue" or soluble specific substance is either identical with, or connected with, the capsule; because, if a certain substance were found to be present in extracts of the capsulated bacillus, and either not present at all or present in very small amounts in similar extracts of the mutant, it would be good evidence that it was indeed the material of the capsule. Of course it does not prove that there are not small amounts of other substances present, such as mucin. In the case of an alkaline extract of an extremely viscid strain of Friedländer's bacillus previously studied by the writer, there was present a large amount of substance or substances containing very little nitrogen in addition to the specifically active carbohydrate.

But in the case of the capsule of the colon strain there was nothing similar, at least not in appreciable amount

In what follows, the special strain of *B. coli*, 1192a, and its mutant 1192b, studied by T. Smith (11–13) was used throughout. In preliminary experiments, in which an alkaline extract of the bacteria was centrifuged and the supernatant precipitated with 2 volumes of 95 per cent alcohol, the precipitate, in the case of the viscid strain, was found to show a white ring very promptly at a dilution of 1 part in 100,000 by weight, with homologous serum. The mutant, non-viscid strain yielded a much less active precipitate, which gave a similar test more slowly and only up to a dilution of 1:1000 by weight, with either serum. It would seem therefore that in one case there was about a hundred times as much precipitable substance as in the other, so it seemed worth while to try to isolate the substance.

Preparation of Soluble Specific or Capsular Substance.—In all about 90 ordinary tin pie plates, each covered with a tin plate of larger size, were poured and inoculated. The ordinary stock veal infusion agar kept on hand in the laboratory was used, with the addition of 0.1 per cent dextrose just before pouring. The agar surfaces were very liberally inoculated with a suspension from 24 hour agar slants in normal saline. After 2 days' incubation, the growth was removed in distilled water (14). In this condition it was still extremely viscous. The bacterial emulsion was diluted to a volume of 500 cc., treated with about 75 cc. of 10 per cent KOH, warmed to 70°, and kept at that temperature about 30 minutes. After neutralizing and making slightly alkaline to litmus, adding about 10 gm. of sodium acetate and 150 cc. of 95 per cent alcohol, a clear supernatant was obtained after centrifugation and the sediment of bacterial bodies rejected. The substance in the supernatant was precipitated on adding 95 per cent alcohol up to 1.2 volumes. It was partially purified by repeated solution and precipitation with 1.2 volumes of 95 per cent alcohol, added drop by drop with mechanical stirring, in the presence of plenty of electrolyte in the form of sodium acetate, usually from solution distinctly alkaline to litmus. Precipitation from acid solution was also employed. The discarded supernatant contained much orange-brown material and very little specific substance as determined by the precipitin test. Precipitation with alcohol was continued 5 to 7 times, usually until the supernatant was practically colorless. The final precipitate, which unlike similar Friedländer material dissolves readily in warm distilled water, was put through a Berkefeld filter, after sufficient dilution, if still turbid. After concentration to convenient volume and reprecipitation with alcohol, it was washed with alcohol in increasing concentration and dried *in vacuo* over sulfuric acid. The yield from 90 plates was somewhat over 2.3 gm. It was active with homologous serum when diluted to 1 part in 2 millions, using the Fornet-Müller ring test.

Description and Analysis.[1]—The biuret test was negative. There was no precipitate (in $\frac{1}{2}$ per cent solutions) with tannic acid, phosphotungstic acid, Esbach's reagent, 5 per cent copper sulfate solution, 10 per cent uranyl acetate solution, or saturated barium hydroxide solution. There was no color with iodine. The Molisch test was strongly positive. But with 10 per cent basic lead acetate there was heavy precipitation and with 10 per cent ferric chloride a fairly heavy precipitate soluble in excess of the reagent.

The white, fluffy powder is readily soluble in hot water. In even a 1 per cent solution it is viscous and opalescent, somewhat resembling starch. It is not stringy, like the original growth on agar. It is very readily hydrolyzed by boiling with acid, and the hydrolysate reduces Fehling's solution, showing that a reducing sugar is present. It gives a slight naphthoresorcinol test for glucuronic acid and a slight test with orcinol, indicating the presence of glucuronic acid, but too faint for a pentose. There is a faint turbidity on boiling with barium hydroxide which is not removed by the addition of hydrochloric acid, thus indicating the presence of a hydrolyzable sulfuric acid. Very likely this is due to an impurity.

The substance, on drying in an Abderhalden dryer at 100°, lost 11.34 per cent of water. The elementary analysis, which was very kindly done by Dr. Elek, was as follows:

Ash 9.24 per cent —
C = 42.26 per cent
H = 5.82 " "
S = 0.6 " "
N = 0.6 " " (my own figure, micro-Kjeldahl, on 10 mg. portion)

Calculated on ash-free basis —
C = 46.56 per cent
H = 6.41 " "
For $(C_6H_{10}O_5)_x$ —
C = 44.4 per cent
H = 6.2 " "

So the carbohydrate nature of the substance is evident.

1 gm. of powder was used in the preparation of an osazone. It was hydrolyzed by boiling in 10 cc. of 2 per cent hydrochloric acid

[1] The writer was greatly assisted by Dr. P. A. Levene in this portion of the work.

under return condenser. The reaction was carefully followed by observing the rotation and the reducing sugar present in the solution from time to time.

Rotation		Reducing power equivalent to glucose
Initial	0.05	—
After ½ hr.	0.06	—
" 1½ hrs.	0.06	800 mg. (about 80 per cent)
" 5½ "	0.08	785 "

Since the values in the above table showed no significant change, heating was stopped, and the hydrolysis mixture neutralized with sodium hydroxide till only slightly acid to litmus. It was filtered and concentrated under reduced pressure to 60 cc. To obtain a phenylosazone it was warmed on the water bath with 2 gm. of free phenylhydrazine dissolved in glacial acetic acid. After 45 minutes on the water bath, it was heated a trifle more and immediately filtered through a folded filter. To this filtrate a second time phenylhydrazine in glacial acetic acid, to the amount of 1 gm., was added and the solution kept on the water bath as long as an osazone was forming. This was again filtered off, and a third precipitate of osazone obtained from the filtrate after standing about 1 hour more. The first lot of osazone was kept separate, but the second and third, which had a similar appearance, were combined. Each precipitate was taken up in methyl alcohol to remove adhering impurities, so that the final osazones were free from oil drops and consisted of long crystalline needles or rosettes.

The melting point of the first was 196°C. The second sintered at 180° and decomposed at 195°. Neither showed optical rotation. The second was analyzed for nitrogen, with the following results: From 0.0620 gm. substance was obtained 8.30 cc. nitrogen at 763.3 mm. Hg and 22°C. This is equivalent to 15.55 per cent nitrogen. (Calculated for $C_{18}H_{22}O_4N_4$, N = 15.63 per cent.)

The fact that the observed rotation of the hydrolysate was very low for the reducing power, and that the osazone seemed to be entirely inactive optically, makes it seem likely that the hydrolysis mixture contained at least two hexoses, one dextro- and the other levorotatory. The osazones were not separable, or they may have been identical. From these facts it seems established that the substance obtained

from the colon bacillus belongs in the class of carbohydrates, as shown by the elementary analysis, that it contains glucuronic acid, and hexoses. The total reducing power of the sugars obtained on hydrolysis calculated as glucose was equivalent to 80 per cent of the weight of the substance. The substance thus differs from all the others reported.

Attempt to Relate the Specific Carbohydrate Obtained from the Capsulated Bacillus to Its Greater Virulence.—The question naturally arose[2] as to whether it were possible to find out how the capsular material acts to increase virulence. Whether it may function as a morphological capsule, or partly dissolve and thus act at a distance by neutralizing the antibody in the blood of the host.

TABLE I.

Guinea pig No.	Weight	Culture	Extract	Result
	gm.	*cc.*	*mg.*	
1	355	0.3	—	Lives
2	350	0.6	—	"
3	350	0.9	—	"
4	360	1.2	—	Dead in 22 hrs.
5	355	0.3	10	Lives
6	350	0.6	10	"
7	350	0.9	10	Dead in 10 hrs.

It seems, from a consideration of the following observations, that in the case of the viscid strain the substance must be acting to a large extent as a closely adherent protecting layer, probably permitting multiplication of the organisms before they can be destroyed. We know from the papers by Smith and associates (11–13) on these mutants that capsulated strains are practically not phagocyted at all. If capsular substance is of significance in the production of virulence it may very likely be in connection with non-phagocytability.

A few experiments were made on guinea pigs to see if the substance extracted from cultures as a carbohydrate had any influence on the course of the disease produced by *B. coli*. The mutant (b) was tried

[2] For suggestions at this point the writer is particularly indebted to Dr. Hans Zinsser and Dr. J. H. Mueller.

first. Table I illustrates the procedure for testing any increase in virulence due to admixture of the living 24 hour bouillon culture with the extract. This was ground up in normal saline. The injection was made into the peritoneal cavity of guinea pigs.

An apparent increase in virulence may be deduced from the table. Several other tests yielded similar data. The (a) form was tried next to see whether it also might be favored by the preparation. 5 mg. of the preparation in normal saline were mixed with graded amounts of the culture and the mixture injected into the peritoneal cavity. The minimum fatal dose was definitely lowered. When, as controls, 5

TABLE II.

1192a and 1192b grown 24 hrs. in flasks containing 75 cc. of veal bouillon.

Serum of rabbit injected with strain	Capsulated strain, 1192a			Mutant, 1192b		
	Filtrate not diluted	Diluted 1/2	Diluted 1/4	Filtrate not diluted	Diluted 1/2	Diluted 1/4
(a)	+*	Tr.	0	+	+	Tr.
(b)	+	0 (?)	0	+	0 (?)	0
1192a and 1192b grown 48 hrs. in exactly similar conditions.						
(a)	+	?	0	+	+	Tr.
(b)	+	0	−	+	0	−
1192a and 1192b grown 3 days in exactly similar conditions.						
(a)	+	+	0	++	+	Tr.
(b)	+	+	0	++	+	0 (?)

* + means a ring that can be seen distinctly. None of these rings were heavy.

mg. of gum tragacanth, and a feebly turbid homogeneous suspension of aleuronat, probably less than 5 mg., were injected with *B. coli*, the same increase in virulence was observed.[3] This method of demonstrating the possible relation of the carbohydrate to virulence was not pursued any farther, since it was evident that some other method would have to be worked out, eliminating mere injury to the peritoneum. The protocols relating to the test with the (a) form are therefore omitted.

[3] See also Benians (15).

It is to be noted that the amount of precipitable substance found in filtrates of early cultures is actually very small, both in the (a) and the (b) forms. This is brought out in Table II.

The two rabbits yielding the highest titer serum, Nos. 1 and 2, had been given intraperitoneal injections, 5 days apart, of increasing amounts of killed growth from 24 hour agar slants emulsified in normal saline. It is impossible to clear the culture of the capsulated strain by centrifuging. An attempt was made to see if any rings could be distinguished in a dilution of 1:8 to eliminate turbidity, but there was not enough precipitable substance to give a reaction at this dilution. So recourse to filtration through a small Berkefeld was necessary. Saline was always passed through first and tested to insure absence of anything giving a ring with the most potent serum (a). Then half the 75 cc. of culture fluid was passed through and rejected, since precipitable substance might be adsorbed by the filter at first. Inasmuch as all the material of all preparations made had been put through Berkefeld V's or N's and there was no trouble with adsorption, the precaution of using only the last part of the filtrate would seem sufficient.

DISCUSSION.

The condition presented by *B. coli* (a) and (b) was quite different from that found by Dochez and Avery (7) when studying the pneumococcus. During the early stages of vigorous growth this organism forms a readily soluble substance which diffuses into the culture medium *in vitro*, and in human and animal infections is present in the blood and urine. The writers could not demonstrate that this substance was responsible for the intoxication occurring in lobar pneumonia, but they were able to state, after the study of 112 cases, that if large amounts were excreted, the outcome was usually fatal. The table on page 479 of Dochez and Avery's paper is particularly instructive. This is from the study of a flask inoculated with an organism of Type III, which forms somewhat more precipitable substance than I or II. After only 4 hours, the undiluted filtrate showed heavy flocculation with homologous serum. A 24 hour culture showed a heavy precipitate at a dilution of 1:30. A trace was recorded at a dilution of

1:120. Evidently the material diffuses out into the culture fluid in considerable amounts.

When we consider the capsulated *B. coli*, we find an entirely different state of things. As shown in the table, there was not a trace of precipitin reaction visible in the case of a 24 hour culture at a dilution of 1:4, nor was there in that of a 3 day culture. When undiluted, there was a good, but not a particularly heavy reaction, even with a precipitating serum of very high titer. Evidently in this case the precipitable substance does not dissolve out into the medium during the growth period. The amount of specific carbohydrate capable of reacting with homologous serum in a 1 to 3 day culture of either (a) or (b) is practically nil.

There remains the question of the relative amounts of precipitable material present in the two strains, the one capsulated and the other not, for this ratio is perhaps at this time the best chemical evidence obtainable of a relation between capsule and soluble specific substance. More crude material is obtainable from equal areas, for example, the surface of ten pie plates in the case of the capsulated strain. No count of the relative numbers of organisms involved has been attempted, but from the uncapsulated strain only about two-thirds as much crude material can be obtained per unit of culture surface. And this material is about 100 times *less active* with high titer precipitating serum (from either strain) than is similar material from the capsulated strain. No attempt has been made to prepare or purify a large amount of the "residue" from the mutant.

The simplest assumption is therefore that suggested by Mueller (8) and Heidelberger, Goebel, and Avery (9), that capsular material and soluble specific substance are identical. In the case of a morphological capsule, the specifically precipitable substance or "residue" probably is produced in much larger quantity and located peripherally Other substances, such as bacterial mucins, may also take part in the capsule formation. In the case of the capsulated *B. coli*, there was no indication of more than a trace of mucin. From the ease with which the material can be obtained in relatively pure condition, it is very unlikely that much is present. On the other hand, in the preparation of specific carbohydrates from a peculiarly viscid strain of Friedländer's bacillus (unpublished) there was obviously a *very*

large admixture of an impurity that was probably mucin, or something similar.

The substance obtained from the capsulated strain of the colon bacillus is not identical with any specific carbohydrate thus far already described. It is composed of 80 per cent of hexose, probably partly of dextro- and partly of levorotatory sugar, since the rotation of the hydrolysate is slight. It is precipitable by basic lead acetate and ferric chloride. It is rather lighter in weight and more readily soluble in distilled water than are some similar substances prepared by the writer from the Friedländer bacillus. The presence of glucuronic acid is indicated. Some evidence for a relation between capsule and specific substance was obtained. From equivalent areas of growth on agar in the case of the mutant about two-thirds as much material, as in the case of the viscid strain, precipitable with 1 volume of alcohol, can be obtained. This material, obviously very impure, is about 100 times *less* active with homologous serum than similar material from the capsulated strain. Although this cannot be interpreted as proving that the capsulated organism contains 100 times as much specific carbohydrate as the mutant, it is a very significant difference.

The specific substance of the capsulated colon strain does not diffuse out into the fluid culture media, as does that of the pneumococcus. There is so little present in the filtrate of a 24 hour culture, such as used in the original virulence tests, that it indicates that the substance functions distinctly as a capsule, a protecting layer, rather than in solution. Attempts to recombine the extract with living bacilli in testing virulence proved unsatisfactory, since non-specific additions in minute amounts also reduced the resistance of the peritoneal cavity of the guinea pigs.

SUMMARY AND CONCLUSIONS.

1. The soluble specific substance obtained from a capsulated strain of *B. coli* is not identical with any specific substance heretofore described. It is a carbohydrate, composed of 80 per cent of hexose, probably partly of dextro- and partly of levorotatory sugar, since the rotation of the hydrolysate is low. Glucuronic acid is probably present in the molecule.

2. Crude "residue" or specific substance obtained from the uncapsulated mutant was about 100 times *less* active with homologous serum than similar material from the capsulated strain. This supports the view that capsular substance and soluble specific substance are the same. In cases where there is a well marked capsule, the specific substance is probably produced in greater amount and located peripherally.

3. Capsular substance is probably significant for virulence when functioning as a morphological capsule. It is present in filtrates of young culture only in very small amounts.

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