CXXXIII. STUDIES IN IMMUNO-CHEMISTRY.

I. THE PREPARATION AND PROPERTIES OF A SPECIFIC POLYSACCHARIDE FROM *B. DYSENTERIAE* (SHIGA).

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THE recognition of the important part played by polysaccharide substances in the specific reactions that are induced by bacterial antigens, such for example as those which take place between virulent forms of bacteria and the homologous antibacterial immune-sera, followed the observation of Dochez and Avery [1917] that culture filtrates from the three main types of *Pneumococcus* contain a soluble substance which gives specific precipitation with the homologous immune-serum. The serologically reactive material was subsequently isolated and identified by Heidelberger and Avery [1923; 1924; 1925] and Avery and Goebel [1933] as a complex polysaccharide which was contained in the bacterial capsule and which was specific for each of the types of *Pneumococcus* investigated. Furthermore, their work indicated that the specific polysaccharides are not antigenic, but that as they exist in the intact bacterial cell they are almost certainly bound with some other substance—most probably a protein—and that it is the resulting complex which possesses antigenic properties.

The conception of antigenic structure which attributes the specific immunological properties of a complete bacterial antigen to a polysaccharide constituent and the antigenicity of the bacterial complex to a combination of this constituent with some other substance is now supported by a considerable body of evidence which, when considered in conjunction with studies on bacterial variation, has gradually led to the idea that the essential difference between the antigen of a virulent and of an avirulent organism is the presence in the former of a specific polysaccharide which is not found in the avirulent strain. Substances, such as the specific bacterial polysaccharides, that do not possess complete antigenic power but have the capacity of reacting with immune-sera prepared against the whole antigen, have been termed haptens by Landsteiner.

As soon as it was generally recognised that the primary function of the polysaccharide portion of a bacterial antigen is to endow the antigen with its characteristic immunological specificity it became of considerable importance to ascertain the influence exerted by certain chemical groupings within the polysaccharide on the specificity of the whole antigenic complex.

Considerable advances have already been made in our knowledge of the influence of the structure and of the stereochemical configuration of simple carbohydrates on the specificity of artificial carbohydrate-protein antigens [Goebel and Avery, 1929; Goebel *et al.*, 1932; 1934], but it must be admitted that as yet we possess little definite insight into the essential factors that are responsible for the strict specificity, or for overlapping specificity where this exists, that is shown by the serologically reactive bacterial polysaccharides. Goebel [1935] attempted to find a chemical basis for the immunological cross-reactions

that are displayed by the specific polysaccharides of *Pneumococcus* Types III and VIII, and his investigations revealed that the polysaccharides of these strains contain an identical uronic acid component and that this molecule is most probably the common structure within each of the polysaccharides which gives rise to the overlapping specificity.

With the view of obtaining further knowledge concerning the nature of the carbohydrate groupings that are associated with immunological specificity in bacterial antigens a study has been made of the specific polysaccharide of *B. dysenteriae* (Shiga). In a previous paper [Morgan, 1931, 1] a preliminary account was given of the isolation of a specific polysaccharide from this organism. Since then a number of modifications in the technique of isolation and purification have been introduced, and these, together with the properties of the purified specific polysaccharide, are described in this communication.

An examination of the properties of the crude, protein-free polysaccharide preparations isolated from Shiga's bacillus by the original process showed that they were mixtures of several polysaccharides [Morgan, 1931, 2]. The introduction of certain modifications in the methods of isolation and purification of the specific polysaccharide has made it possible to separate at least three non-specific polysaccharides from their association with the specific substance. Two of these polysaccharides give specific precipitation with an anti-agar serum up to a very high dilution and, therefore, appear to be related to the agar on which the dysentery bacillus was grown. An examination of these substances has shown that they do not consist of unchanged agar but that they are most probably soluble agar derivatives which still retain the serologically reactive configuration of groups that are present in the undisrupted agar molecule. It seems probable that these soluble forms of agar have been produced by certain enzymic processes associated with the growth of the dysentery bacillus. Another non-specific polysaccharide substance, which was found to be nitrogen-free and strongly dextrorotatory, was subsequently identified as glycogen.

EXPERIMENTAL.

The isolation of the specific polysaccharide.

The variant of *B. dysenteriae* (Shiga) that has been used throughout this work corresponds to the "smooth" form originally described by Arkwright [1921]. The organism yields a stable suspension in ordinary broth culture and in 2.5% saline, and it produces typical smooth, glistening colonies when grown on nutrient agar.

The bacilli from 250 Roux bottles were washed off with distilled water saturated with chloroform in the proportion of about 1 l. to 100 bottles. The final bacterial suspension was adjusted to p_H 4.0 with acetic acid and was immediately passed through a Sharples centrifuge, the clear supernatant was rejected and the organisms were resuspended in about one-tenth of the original volume of distilled water and stored at 0° in the presence of chloroform. This procedure was repeated until the growth from 1000 Roux bottles had been collected. The rejection of the first supernatant fluid, which contained most of the soluble products of bacterial metabolism, together with a proportion of the specific substance, eliminated practically the whole of the non-specific polysaccharides that made up so large a part of the protein-free mixed polysaccharide preparations which were isolated by the original method. The washed and resuspended bacteria were adjusted to p_H 4.0 by the addition of acetic acid and were heated at 100° for about 45 min. The suspension was again passed through

the supercentrifuge or filtered through chain-cloth to remove the bacterial debris and the coagulated bacterial protein. The process of extraction was repeated until the whole of the specific polysaccharide in the bacterial mass had been recovered. The method of isolation of the specific polysaccharide from the combined acid extracts was essentially the same as that which was described in the earlier communication. Since that time, however, the precipitation of the specific substance as a basic lead complex has always been carried out at 0° and the addition of the dilute ammonia carefully controlled in order to avoid an excess of alkali. The basic lead complex was immediately filtered off, washed with ice-cold water, resuspended in water and saturated with carbon dioxide. If the original supernatant culture fluid has been completely removed from the bacilli before they are heated in the acetic acid solution, only a small precipitate is produced when mercuric acetate is added to the acidified polysaccharide solution resulting from the decomposition of the basic lead complex with carbon dioxide. The addition of mercuric acetate at this stage to some preparations produced no precipitate. The test must be repeated on a small sample of the solution after it has been concentrated by evaporation to about one-tenth of its original volume; only if the test is then negative can the mercury purification be safely omitted from the main preparation. The final material, which was found to be protein-free, consists of the specific bacterial polysaccharide hapten contaminated with small amounts of various non-specific polysaccharides the last traces of which were removed as follows. The crude polysaccharide material was dissolved in ten parts by weight of water and glacial acetic acid was slowly added until a concentration of 90 % acetic acid was reached. The precipitate which formed possessed a relatively low specific activity and was removed by centrifuging. Acetic acid was then added to the clear supernatant fluid until the whole of the specific polysaccharide was precipitated; under these conditions this occurs between 90 and 96 % concentration of acetic acid. The material which remained in solution and which possessed only slight serological activity was recovered by the addition of several volumes of alcohol or ether, which precipitated it as a white flocculent mass. The specific polysaccharide substance was further purified by solution in 10 parts of water and treatment with acetic acid as described above until a 10 % aqueous solution of the substance gave no precipitate on the addition of ten times its volume of glacial acid; precipitation should, however, be complete when a further 10 parts of acetic acid are added. The specific material was again dissolved in 10 parts of water and was fractionated by the addition of acetone which was slowly added with constant shaking. A sticky oily precipitate first formed and was separated after the solution had remained at 0° overnight. The further addition of acetone produced a flocculent precipitate which could be readily separated by centrifuging and a final fraction was obtained by pouring the supernatant fluid into several volumes of acetone. The fractional precipitation of the crude material with acetic acid and subsequently with acetone removes all the non-specific polysaccharide substances. A preparation of the specific polysaccharide is not considered homogeneous until the first and last fractions obtained from the use of both these methods have been found to possess identical chemical and serological properties.

The final product, after several reprecipitations from aqueous or pyridine solution by alcohol, had $[\alpha]_D + 98^\circ$ ($c = 1.0\%$) and repeated fractionation from aqueous or pyridine solution with acetic acid, acetone, alkaline, neutral or acid alcohol failed to yield a polysaccharide fraction which possessed a different specific rotation or serological activity. The specific polysaccharide preparations isolated from two different strains of Shiga's bacillus were found to be identical

in all the chemical and immunological properties examined. During the course of this work more than 10,000 Roux bottles of growth have been investigated by the method described and the results have shown that from 1000 Roux bottles 1–2 g. of pure specific polysaccharide are usually obtained. The specific polysaccharide has been found to represent not more than about 20% of the total polysaccharide material present in the original unwashed bacterial suspension.

Chemical properties of the specific polysaccharide.

The specific polysaccharide, $[\alpha]_D + 98^\circ$, after repeated precipitation from aqueous solution by alcohol or acetone, is obtained as a white amorphous substance. It can be heated at 100° in air or *in vacuo* without becoming discoloured. Analysis of the substance after drying at 100° over phosphorus pentoxide *in vacuo*: C, 41.9; H, 6.35; N, 1.61%. The polysaccharide yields 97% of reducing substances (Hagedorn and Jensen) calculated as glucose after acid hydrolysis. A specimen of the polysaccharide (0.64 g.) required 7.1 ml. 0.01 *N* caustic soda for neutralisation; the acid equivalent is therefore about 9000. An aqueous 2% solution of the polysaccharide forms no precipitate when it is treated with solutions of the following reagents: tannic acid, barium hydroxide, mercuric acetate, uranium acetate, copper sulphate and lead acetate. The orcinol and phloroglucinol tests for pentoses, the naphthoresorcinol test for glycuronic acid and the salicylsulphonic acid, trichloroacetic acid and picric acid tests for proteins were uniformly negative when tested on 20 mg. samples of the specific substance dissolved in 0.5 ml. of water. The polysaccharide gives no coloration with Selivanoff's reagent or with iodine solution. It is not precipitated when warmed with Fehling's solution. After prolonged electrodialysis, using cellophane membranes and a potential of 120 v., the polysaccharide which was recovered was indistinguishable, in all the chemical and immunological properties examined, from the untreated substance. The specific polysaccharide dialyses very slowly through a cellophane membrane; the rate of diffusion however is too small to be of any practical value. A 1% solution of the polysaccharide in acetic acid, p_H 2.5, is serologically active after being heated for several hours at 100° . The specific substance is slightly soluble in a saturated solution of ammonium sulphate and this property has occasionally been utilised as an alternative method for the removal of non-specific polysaccharide impurities.

In an earlier paper it was shown that treatment of the crude polysaccharide preparation with nitrous acid, under conditions similar to those used in Van Slyke's technique for the determination of amino-groups in amino-acids, failed to liberate the nitrogen contained in the specific polysaccharide complex. A similar experiment has now been made with a specimen of the pure polysaccharide; although the reagents were allowed to react for 18 hours at 35° there was no liberation of nitrogen nor was there any demonstrable alteration in the serological properties of the specific substance. Hynd and Macfarlane [1926] found that the action of nitrous acid on certain nitrogenous sugar derivatives could be considerably accelerated by the addition of mineral acid to the reacting mixture. A preliminary experiment showed that the prolonged action of sulphuric acid of higher concentration than 0.1 *N* caused destruction of the specific serological properties owing to hydrolysis of the polysaccharide. The reaction mixture was, therefore, made 0.05 *N* with sulphuric acid and allowed to react at 35° for 18 hours. The results given in Table I show that the nitrous acid has no action on the specific substance.

These experiments indicate that the nitrogen contained in the polysaccharide is most probably not present in the form of free amino-groups. This conclusion is

Table I.

The result of the action of nitrous acid on the specific polysaccharide in presence of 0.05 *N* H₂SO₄ at 35° for 18 hours.

Property	Before treatment	After treatment
Specific rotation,* $[\alpha]_D$	+95°	+93°
Specific serological activity	1 : 12 × 10 ⁶	1 : 12 × 10 ⁶
Nitrogen (%)	1.62	1.70
Minimum anaphylactic dose (mg.)	0.015	0.010
Reducing power (H. and J.) (%)	2.4	2.2

* Uncorrected for ash.

rendered more certain when the stability of the nitrogen atoms to the action of alkali is considered. It has been found that whereas glucosamine and chondrosamine—carbohydrate substances which contain free amino-groups—yield practically the whole of their nitrogen in the form of ammonia on heating for a few minutes with normal alkali at 100°, the specific polysaccharide behaves under these conditions like the *N*-substituted substances, acetylglucosamine and acetylchondrosamine, which evolve only a small proportion of their nitrogen. Further evidence for the existence in the specific polysaccharide of substituted amino-groups only will be given when the results of the acid hydrolysis experiments are considered.

The action of emulsin, malt-diaxase, takadiastase and a commercial preparation of cellulases and hemicellulases called "Luizyme" on an aqueous solution of the specific polysaccharide was investigated. In no case was there any detectable decomposition of the substance as determined by an increase in reducing power or by the usual serological precipitation technique. The decomposition and inactivation of the specific polysaccharide by a species of *Myxococcus* has already been described [Morgan and Thaysen, 1933].

Acetylation of the polysaccharide.

The thoroughly dried and finely powdered polysaccharide (0.50 g.) was added to a mixture of freshly distilled pyridine (16 ml.) and "A.R." acetic anhydride (10 ml.). The material dissolved after 5 minutes' heating at 50° to yield a pale yellow solution; the heating was continued for 1 hour at 70°. The pyridine solution was then cooled and poured into 250 ml. of ether. The fine buff-coloured precipitate of the acetyl compound was filtered off, washed successively and thoroughly with water (200 ml.) and alcohol-ether and dried *in vacuo* over sulphuric acid. The aqueous washings failed to give specific precipitation when they were diluted 1 : 100 or 1 : 1000 with saline and were mixed with an equal volume of a 1 : 4 dilution of a *B. dysenteriae* (Shiga) serum; it seemed therefore that the whole of the specific polysaccharide had been converted into the insoluble acetyl compound. The acetylated substance was dissolved in acetone (8 ml.), filtered to remove traces of insoluble material and re-precipitated by pouring into a mixture of 100 ml. of absolute alcohol and 50 ml. of dry ether. The solution of the substance in acetone and its reprecipitation with alcohol-ether mixture were repeated several times. Fractional precipitation of the acetyl polysaccharide from acetone or chloroform solution by the addition of ether showed that the derivative was homogeneous. The acetyl polysaccharide was obtained as a white amorphous solid; it was insoluble in methyl and ethyl alcohol, ether and water, but was readily soluble in chloroform and acetone; yield 0.66 g.; $[\alpha]_D^{25} + 71^\circ$ (in acetone, *c* = 0.5 %). Analysis: C, 48.0; H, 5.4 %.

Regeneration of the polysaccharide. The acetylated polysaccharide (0.6 g.) was dissolved in 30 ml. of aqueous acetone, and a slight excess of *N* KOH was added and the mixture kept at 37° for 4 hours. A test for serological activity showed that after this time the acetyl derivative had been hydrolysed and that the specific polysaccharide had been liberated. The solution was dialysed against distilled water until it was free from acetone and salts, evaporated to a volume of 10 ml. and poured into several times its volume of alcohol containing a trace of potassium acetate. The precipitated polysaccharide was filtered off, washed with absolute alcohol and dried *in vacuo*. The regenerated substance was very similar in chemical and immunological properties to the original polysaccharide. It gave specific precipitation with the homologous immune-serum at a dilution of 1 in 10⁷ and induced fatal anaphylactic shock when it was given intravenously to guinea-pigs that had been passively sensitised with immune-serum. The polysaccharide had $[\alpha]_D^{180} + 95^\circ$ and was insoluble in methyl and ethyl alcohol, acetone and chloroform. Analysis: C, 42.1; H, 6.5; N, 1.6 %.

Benzoylation of the polysaccharide.

The benzoylation was carried out by the method of Schotten and Baumann. To an aqueous solution of the polysaccharide (0.45 g. in 20 ml. water) benzoyl chloride was slowly added with vigorous stirring; the solution was kept slightly alkaline during the reaction by the addition of dilute sodium hydroxide. The benzoylated polysaccharide separated from the solution as a heavy white precipitate. After the reaction was complete a 1 in 100 dilution of the clear fluid failed to give a positive Molisch test or to give specific precipitation with a *B. dysenteriae* (Shiga) immune-serum. The precipitate of the benzoyl compound was filtered off, washed successively with 0.01 *N* NaOH, water and alcohol and dried *in vacuo* over sulphuric acid. The white amorphous material was dissolved in 5 ml. of 95 % acetone, centrifuged to remove traces of insoluble residue and reprecipitated from the acetone solution by the addition of 200 ml. of a mixture of equal parts of alcohol and ether, $[\alpha]_D^{160} + 63^\circ$ (in acetone).

The regeneration of the polysaccharide. An aqueous acetone solution of the benzoyl polysaccharide was treated with alkali exactly as described above for the regeneration of the polysaccharide from the acetate. The properties of the regenerated polysaccharide were similar to those found for the original material.

The serological properties of the specific polysaccharide.

The specific polysaccharide gives a definite precipitate up to a dilution of 1 : 12,000,000 with the homologous immune rabbit or horse sera but it fails to form a precipitate when it is mixed with a normal rabbit or horse serum, with a horse serum that contains a high titre of agar precipitins or with an immune rabbit serum which had been produced by the intravenous injection of formalised "rough" *B. dysenteriae* (Shiga). Serial dilutions of a homologous immune rabbit serum which had been repeatedly adsorbed with a suspension of washed "rough" *B. dysenteriae* (Shiga) gave precipitation to the same end-point as the unadsorbed serum when they were treated with an optimum concentration (1 : 100,000) of the specific polysaccharide. From these observations it would appear that the specific polysaccharide is not associated with the antigenic complex that is present in the "rough" variant. The absence of the serologically active polysaccharide from the carbohydrate material that has been isolated from the "rough" variant of Shiga's bacillus gives support to this conclusion. In order to obtain evidence that the specific polysaccharide, in the form in which it

has been isolated, accounts for all the serological phenomena of specificity associated with the undisrupted antigenic complex of the "smooth" form of *B. dysenteriae* (Shiga), the following experiments were made. A homologous antibacterial rabbit serum (10 ml.), which had been repeatedly adsorbed with a dense washed suspension of "rough" *B. dysenteriae* (Shiga), was treated at 37° with a 1:5000 dilution of the specific polysaccharide until the serum no longer produced a precipitate on the subsequent addition of the polysaccharide solution. The serum was kept at 0° for 24 hours and filtered at this temperature through a Berkefeld filter candle. The addition of a further quantity of the specific substance produced no precipitate after the adsorbed serum had stood for 2 hours at 37° and overnight in the cold room. Two other immune-sera were treated in the same manner; untreated samples of the three immune-sera were used as controls.

A dense freshly prepared suspension of *B. dysenteriae* (Shiga) was divided into three (50 ml.) portions which were adjusted to p_H 4.5, 7.0 and 8.5 respectively by the addition of an equal volume of an appropriate phosphate buffer solution. The bacterial suspensions were again divided into equal (50 ml.) parts. Three of the portions, at p_H 4.5, 7.0 and 8.5 respectively, were heated at 100° for 10 min. and filtered at once through small sterile Seitz filters. The three remaining portions were allowed to autolyse at 37° in the presence of toluene for 72 hours and were then filtered in the same manner. A series of ten-fold dilutions in saline of each of the six filtrates was tested against the adsorbed and unadsorbed antibacterial sera. The results obtained with each set of three serum samples were identical; one series of results is given in Table II. It will be seen that

Table II.

The results obtained when extracts of *B. dysenteriae* (Shiga) are added to an antibacterial serum from which the precipitins for the specific polysaccharide and the "rough" antigenic complex had been removed by adsorption.

Treatment of <i>B. dysenteriae</i> (Shiga) sus- pension	<i>p</i> _H	Dilution of filtrate tested							
		1:10	1:100	1:1000	1:5000	1:10,000	1:10	1:100	1:1000
		Unadsorbed serum					Adsorbed serum		
Heated 100° 10 min.	4·5	##	##	+	+	tr	tr	0	0
	7·0	##	+	+	0	0	0	0	0
	8·5	##	+	+	tr	0	+	0	0
Autolysed 35° 72 hours	4·5	##	+	+	+	0	0	0	0
	7·0	##	+	+	tr	0	tr	0	0
	8·5	##	##	+	+	tr	+	0	0
##, very heavy precipitation.					#, heavy precipitation.				
+, distinct precipitation					tr, trace of precipitation.				

in no instance was there any appreciable precipitation with the adsorbed serum, a result which indicates that simple extracts or autolysates of the bacterial cell made at an acid, neutral or alkaline reaction do not contain a substance which will precipitate with a serum which has been thoroughly adsorbed with the purified specific polysaccharide and the "rough" antigenic complex. Furthermore, the unadsorbed sera agglutinated suspensions of *B. dysenteriae* (Shiga) to a titre of about 1 in 4000 but after precipitation with the purified specific polysaccharide complete agglutination failed to occur at a higher dilution than 1:10. It appears therefore that treatment of an antibacterial *B. dysenteriae* (Shiga) serum with the specific polysaccharide until the whole of the specific

precipitins for this polysaccharide hapten have been removed also completely removes the agglutinins for the intact homologous organisms.

The specific polysaccharide has been found to possess the property of neutralising the haemolytic action of 'Shiga' heterophile antibody on sheep red-cells as well as of combining with the homologous antibacterial immune-substance. The property of neutralising these two distinct types of antibody suggests that the 'Shiga' polysaccharide contains in each of its molecules two kinds of determinant receptors of diverse specificity [Meyer and Morgan, 1935].

Acid hydrolysis of the specific polysaccharide.

The specific polysaccharide is slowly hydrolysed by dilute mineral acid with the liberation of 97 % by weight of reducing substances calculated as glucose and with the complete loss of its specific immunological properties. Fig. 1 shows the results of a hydrolysis experiment with 0.1 *N* sulphuric acid at 100°. The progress

Fig. 1. *A*, reducing power (Hagedorn and Jensen); *B*, reducing power (hypoiodite method).

of the hydrolysis was followed by estimating the reducing power of the solution by the Hagedorn and Jensen method and by the sodium hypoiodite method as modified by Macleod and Robison [1929]. The reducing power determined by the latter method is, in the absence of certain groups within the molecule, a measure of the free aldehyde groups liberated during the course of the hydrolysis. It will be seen from the curves that during the early stages of the hydrolysis the H. and J. reducing power was almost twice as great as the reducing power of the solution towards the alkaline solution of iodine. The iodine reduction, however, rises more rapidly towards the end of the hydrolysis than the H. and J. reduction and, after about 10 hours' heating, is equal in amount to the reducing power as determined by the latter method. The simplest explanation of the results is that early during the hydrolysis the polysaccharide complex is easily and rapidly broken down

into smaller structural units. The existence of reducing oligosaccharides in the solution during the preliminary stages of the hydrolysis would give rise to a set of reducing values similar to those recorded in Fig. 1 for the hydrolysis products of the polysaccharide. The further action of the acid finally results in the complete breakdown of the intermediate structures into simple hexoses.

From the analytical figures obtained for the purified polysaccharide and from other evidence already mentioned it would appear that the whole of the nitrogen present in the complex is in the form of substituted amino-groups and that most probably one of these groups is associated with one out of every five hexose units which constitute the polysaccharide structure.

The following experiment was made in order to identify the nature of the substituent group attached to the amino-group in the specific polysaccharide. A specimen of the polysaccharide (0.9 g.) which had been repeatedly reprecipitated, first from weakly alkaline alcohol and then from neutral alcohol, was dissolved in 10 % sulphuric acid (25 ml.) heated at 100° for an hour and distilled in a current of steam. The distillate was allowed to run into an aqueous suspension of freshly prepared silver carbonate which was contained in a closed receiver kept at 0°. When the distillation was complete the suspension was warmed slightly, the excess of silver carbonate was removed, the filtrate was evaporated *in vacuo* to 5 ml. and 15 ml. of alcohol were added. After standing at 0° for 18 hours a mass of colourless needles separated; they were filtered off and again crystallised from 75 % alcohol. Analysis (material dried *in vacuo*). (Found: C, 14.5; H, 2.3; Ag, 64.2 %: silver acetate requires C, 14.4; H, 1.8; Ag, 64.7 %). The crystals appeared to be identical with an authentic specimen of silver acetate which had been crystallised under similar conditions. From this experiment the *N*-substituted hexosamine present in the polysaccharide is identified as a *N*-acetylhexosamine. The quantitative estimation of acetyl groups was made according to the method of Pregl and Soltys [1929]. (Found: 4.92 and 5.09 % CO.CH₃. Calculated for a polysaccharide composed of units each of which consist of four hexoses combined with a molecule of acetylaminohexose, CO.CH₃, 5.03 %.) Furthermore the results indicate that the whole of the nitrogen contained in the polysaccharide (1.61 %) is present in the form of acetylated amino-groups.

During the acid hydrolysis of the polysaccharide with 0.1 *N* sulphuric acid there is a gradual elimination of the acetyl groups from the *N*-acetylhexosamine molecules with the consequent formation of primary amino-groups which react with iodine in alkaline solution and give rise to false reducing figures. This is clearly seen from the iodine reduction curve shown in Fig. 1 where the reduction calculated as glucose rises towards the end of the hydrolysis to a value well over 100 %.

In an attempt to follow the rate of liberation of *N*-acetylhexosamine during the acid hydrolysis of the polysaccharide, Morgan and Elson [1934] developed a method whereby this compound could be quantitatively determined in the presence of the mixed hydrolysis products. It was soon found, however, that the *N*-acetylaminohexose which was liberated during hydrolysis with 0.1 *N* sulphuric acid was readily converted by the further action of the acid into the corresponding deacetylated aminohexose. In order to follow this additional hydrolytic change it became necessary to devise a quantitative procedure for the determination of aminohexoses. The work of Pauly and Ludwig [1922] had already shown that glucosamine could be converted into a pyrrole derivative by heating with an alkaline solution of acetylacetone so that it was only necessary to determine the optimum conditions for the quantitative conversion of glucos-

amine or chondrosamine into their corresponding pyrrole derivatives in order to elaborate a method for the determination of these two aminohexoses or presumably any other simple 2-aminohexose. The method, which has already been described in detail [Elson and Morgan, 1933], has enabled the whole of the nitrogen present in the final hydrolysis products of the polysaccharide to be identified as aminohexose. By the use of these methods for the determination of *N*-acetylaminohexose and aminohexose at different stages of the hydrolysis it has been found that during the early stages of the acid hydrolysis of the specific polysaccharide the products, after heating for a few minutes in alkaline solution, develop a reddish purple coloration following the addition of hydrochloric acid-*p*-dimethylaminobenzaldehyde in acetic acid solution. The unhydrolysed and the completely hydrolysed polysaccharide fail to develop the coloration under the same conditions. Furthermore, the treatment of the final hydrolysis products with an alkaline solution of acetylacetone causes a bright red colour to appear when an alcoholic hydrochloric acid solution of *p*-dimethylaminobenzaldehyde is added. The unhydrolysed polysaccharide does not give the coloration when it is treated under similar conditions. The above experimental observations can be readily explained as being due to the presence of *N*-acetylhexosamine units (Formula 1) in the undisrupted polysaccharide complex. The inability of the unhydrolysed polysaccharide to develop the reddish purple colour is due to the absence of the terminal aldehyde group from the *N*-acetylhexosamine units owing to the involvement of these groups in the glycosidic linkages which join the acetylhexosamine molecules to their neighbouring hexoses. It has been found that 1-methyl-*N*-acetylglucosamine, which likewise does not possess a free reducing group, also fails to give rise to the red-coloured complex. During the early stages of the hydrolysis, however, the polysaccharide breaks down into a number of smaller structural units among which single hexoses may exist. In any event, the *N*-acetylhexosamine molecule (II) is now found to possess a reducing aldehyde group as shown in structure (III), which, in the presence of

alkali, presumably changes to the enolic structure (IV) and eliminates water with the formation of a heterocyclic ring structure (V) or (VI), and it is the 2:4-disubstituted oxazole structure (V) or oxazoline structure (VI) which condenses with the *p*-dimethylaminobenzaldehyde and gives rise to the reddish-purple coloration (unpublished observations).

When, however, hydrolysis of the polysaccharide is complete, the acetyl groups are found to be completely eliminated from the *N*-acetylhexosamine molecules with the result that the formation of a heterocyclic ring structure similar to that suggested in the above scheme cannot take place and therefore no coloration develops with the *p*-dimethylaminobenzaldehyde reagent. If the resulting deacetylated hexosamine is now heated with an alkaline solution of acetylacetone, condensation is able to take place with the formation of 2-acetyl-3-methyl-5- α : β : γ : δ -tetrahydroxybutylpyrrole (VII) which readily condenses with *p*-dimethylaminobenzaldehyde to yield the red-coloured complex. It will be seen that with the unhydrolysed polysaccharide the formation of a substituted pyrrole derivative similar to (VII) cannot occur owing to the absence of a primary amino-group in the molecule. The presence of a reducing group in position 1 of the aminohexose does not appear to be necessary for the condensation to take place since it has been observed that 1-aminoglucose readily condenses with acetylacetone to form a pyrrole derivative.

The destruction of the specific immunological properties of the polysaccharide during hydrolysis.

Table III shows that after heating a 1 % solution of the polysaccharide with 0.1 *N* sulphuric acid for 60 min. at 100°, the serological property of forming a specific precipitate when it is mixed with an antibacterial 'Shiga' rabbit serum

Table III.

Showing the destruction of the serological activity of the specific polysaccharide during the course of hydrolysis with 0.1 *N* sulphuric acid at 100°.

Time min.	Dilution of polysaccharide giving specific precipitation with antibacterial rabbit serum								
	2×10^3	2×10^4	1×10^5	2×10^5	1×10^6	2×10^6	6×10^6	12×10^6	15×10^6
0	—	+	++	++	+	+	+	+	±
15	—	+	+	+	+	+	tr	0	0
30	—	+	+	+	tr	0	0	0	0
45	—	+	tr	0	0	0	—	—	—
60	tr	tr	0	0	0	—	—	—	—
90	tr	?tr	0	0	—	—	—	—	—

Signs as in Table II.

has been completely destroyed. From the acid hydrolysis curve, Fig. 1, it will be seen that after 60 minutes' heating, the reducing power of the solution had risen to about 36 % as measured by the H. and J. method, and 18 % as measured by the hypiodite method; both these figures are calculated on the weight of the polysaccharide. These results indicate that as soon as the polysaccharide complex is split into smaller molecules, the specific serological activity, as measured by the precipitation test, disappears. It has been found, however, that the acid hydrolysis products of the polysaccharide at this stage of the hydrolysis cause a specific inhibition of precipitation when they are added to an antibacterial "Shiga" rabbit-serum before the addition of the intact specific polysaccharide.

Furthermore the hydrolysis products, when injected intravenously into guinea-pigs which had been passively sensitised with an antibacterial *B. dysenteriae* (Shiga) serum, caused the animals to become desensitised so that a subsequent injection of unchanged polysaccharide which would normally bring about a rapid and fatal anaphylactic shock, failed to induce anaphylaxis.

The inhibition experiments were carried out as follows. The partial hydrolysis products were neutralised with dilute alkali and diluted with physiological saline to yield a 0.1% solution. An equal volume (0.25 ml.) of immune-serum was added to several different dilutions of this stock 0.1% solution and the mixtures were incubated at 37° for 2 hours. To each mixture 0.25 ml. of a 1:10⁵ dilution of the specific polysaccharide was then added and the tubes were again kept at 37° for 2 hours. After standing at 0° overnight the tubes were examined for the presence or absence of a specific precipitate. In order to determine more closely the point at which the power of the polysaccharide to form a precipitate with immune-serum is completely destroyed, a number of hydrolysis experiments were carried out using different acid concentrations. When 0.01 *N* hydrochloric acid was used the polysaccharide after heating for 16 hours at 100° failed to precipitate with immune-serum but inhibited precipitation and anaphylaxis more efficiently than after it had been heated with 0.1 *N* sulphuric acid at 100° for 1 hour.

Attempts have been made, by means of simple dialysis experiments, to separate the active constituent of the hydrolysis product which is able to cause the specific inhibition of the combination of the polysaccharide with its homologous antibody. The results of inhibition tests carried out with the dialysate and with the bag contents of a typical dialysis experiment through cellophane are shown in Table IV. It is evident that both the non-diffusible bag contents and

Table IV.

Inhibition of the homologous antigen-antibody reaction by the partial hydrolysis products of the specific polysaccharide.

Material	Concentration mg. per 1 ml.	Final dilution of the acid hydrolysis products						
		1 : 750	1 : 1500	1 : 2250	1 : 3000	1 : 3750	1 : 4500	1 : 5250
		Degree of inhibition						
Bag contents	3.3	c	c	c	i	tr	o	o
Dialysate	4.2	c	c	c	ac	i	o	o
c, complete inhibition.		tr, trace of inhibition.						
ac, almost complete inhibition.		o, no inhibition.						
i, incomplete inhibition.								

the dialysate completely inhibit the formation of a precipitate even when the unchanged polysaccharide is added in optimum concentration (1:10⁵). Dilutions of the hydrolysis products greater than 1 : 3000 cause only a partial inhibition of precipitation.

By the use of 0.01 *N* hydrochloric acid it is possible to avoid the elimination of the acetyl group from the *N*-acetylhexosamine during the hydrolysis and, at the same time, to cause the destruction of the whole of the glycoside linkages that exist between the potential aldehyde groups of the *N*-acetylhexosamine units and the neighbouring hexoses. After 16 hours' heating practically the whole of the *N*-acetylhexosamine in the polysaccharide can be estimated by the colorimetric method of Morgan and Elson, a result which indicates that the acetylhexosamine has been liberated in the form of single molecules or, as is more probable, as reducing end-units in the oligosaccharides which have been formed.

A more detailed account of the acetylhexosamine and hexoses that are liberated during the acid hydrolysis of the polysaccharide will be given in a later communication.

The presence of agar-like substances in the crude specific polysaccharide preparations.

During some experiments which were made in 1931 and in which the specific inhibition caused by the partial hydrolysis products of the specific polysaccharide material was first demonstrated, it was noticed that the hydrolysis products continued to yield a precipitate when they were treated with a *B. dysenteriae* (Shiga) horse serum although no precipitate was formed when the corresponding rabbit serum was employed. The precipitation tests that were set up with immune rabbit serum showed that the specific serological properties of the polysaccharide were rapidly destroyed whereas the precipitation tests carried out with the immune horse serum indicated that after a rapid fall in activity during the first 30 min. the precipitating activity of the specific polysaccharide appeared to remain constant at a level of about 1 : 500,000. The results of the precipitation tests are shown in Tables V and VI.

Table V.

Precipitation tests using an antibacterial *B. dysenteriae* (Shiga) rabbit serum.

Time of heating hours	Final dilution of partially hydrolysed crude bacterial polysaccharide preparation (0.25 ml.)						
	1×10^4	5×10^4	1×10^5	5×10^5	1×10^6	3×10^6	6×10^6
0	+	+	+++	+++	+	+	+
0.25	+	+	+++	+	+	+	0
0.50	+	+	+	+	+	0	0
0.75	+	+	+	0	0	0	0
1.0	+	tr	0	0	0	0	0
1.5	?	0	0	0	0	0	0

Signs as in Table II.

Table VI.

Precipitation tests using antibacterial *B. dysenteriae* (Shiga) horse serum (commercial sample).

Time of heating hours	Final dilution of partially hydrolysed crude bacterial polysaccharide preparation (0.25 ml.)						
	1×10^4	5×10^4	1×10^5	5×10^5	1×10^6	3×10^6	6×10^6
0	+++	+++	+++	+	+	+	+
0.25	+++	+++	+++	+	+	tr	0
0.50	+++	+++	+++	+	+	0	0
0.75	+++	+++	+	+	tr	0	0
1.0	+++	+	+	+	tr	0	0
1.5	+++	+	+	+	tr	0	0
2.0	+++	+	+	+	0	0	0

Signs as in Table II.

At that time it was not possible to explain these experimental results, but the observations of Sordelli and Mayer [1931], who found that agar adsorbed on bacteria is rendered antigenic and produces agar antibodies in animals that are immunised with these organisms, suggested that the antibacterial horse serum that had been employed by us as a precipitating serum contained a high titre of agar precipitins.

An examination of several samples of anti-dysentery horse serum showed that they possess a considerable titre of anti-agar bodies and yield precipitates with high dilutions (1 : 500,000) of agar. Anti-dysentery serum is frequently produced on the large scale for therapeutic use by the intravenous inoculation of horses with killed cultures of *B. dysenteriae* (Shiga) which have grown on nutrient agar. During the immunisation agar precipitins are slowly formed and after several courses of injections, which would normally extend over several months, a potent anti-agar serum is produced. The absence of agar antibodies from the immune rabbit serum is presumably due to the short course of immunisation which they receive as compared with the intensive and repeated series of inoculations usually given to horses that are producing therapeutic sera for general use. It has been observed that for the production of a precipitating anti-meningococcus serum in rabbits it is necessary to administer a very prolonged course of immunisation and under these circumstances the resulting rabbit serum has occasionally been found to contain agar-precipitins.

The anti-agar horse serum that has been used in the experiments described in this paper was obtained from a horse that had been immunised with agar-grown meningococci and was therefore free from precipitins which would react with the specific polysaccharide of *B. dysenteriae* (Shiga).

It follows from these serological observations that the specific polysaccharide preparations in use at that time were contaminated with agar-like polysaccharide substances and therefore reacted with the anti-agar bodies present in the immune horse serum. In our experience specific polysaccharide preparations which have been obtained from organisms that have grown on agar always react with the formation of a precipitate when they are mixed with an anti-agar immune serum unless a very thorough chemical purification has been carried out.

THE NON-SPECIFIC POLYSACCHARIDES.

The isolation and identification of the various non-specific polysaccharide substances present in the supernatant fluid obtained from the original bacterial suspension has been considered to be of subsidiary importance in this investigation and for this reason they have only been superficially examined and will only be briefly discussed.

The first supernatant fluid, after the bacilli had been removed, was treated with normal and basic lead acetate and with mercuric acetate in the same manner as has been described for the isolation of the specific polysaccharide. From the supernatant fluid obtained from one batch of bacterial growth (1000 Roux bottles) about 10 g. of mixed, protein-free, non-specific polysaccharide was obtained. Fractional precipitation of the mixture with acetic acid yielded several fractions, the $[\alpha]_D$ of which varied from -60 to $+20^\circ$. The further fractional precipitation of an aqueous solution of each of these fractions, first with acetic acid and finally with acetone has yielded three apparently homogeneous polysaccharide substances; their properties are shown in Table VII.

Table VII. *The properties of the non-specific polysaccharides.*

Polysaccharide	$[\alpha]_D$	Nitrogen %	Greatest dilution of fraction giving precipitate with	
			<i>B. dysenteriae</i> (Shiga) serum	Anti-agar serum
1	-60°	5.2	0	1 : 5,000,000
2	$+20$	0.6	0	1 : 10,000,000
3	$+195$	0.0	0	0

Polysaccharide fraction no. 3 has been identified as glycogen. Since the polysaccharides 1 and 2 were not exhaustively fractionated it is not possible to claim that the specific rotations are those of pure substances. A strongly laevorotatory substance, $[\alpha]_D - 78^\circ$, which is readily soluble in 96% acetic acid and which possesses a very high nitrogen content (14.1%) has also been isolated.

A detailed account of the agar-like polysaccharides that are associated with the specific polysaccharide of *B. dysenteriae* (Shiga) will be published later.

DISCUSSION.

The serological behaviour of the purified specific polysaccharide isolated from *B. dysenteriae* (Shiga) supports the view that this substance is responsible for the most important specific immunological properties of Shiga's bacillus and that it represents a further example of the class of bacterial substances, termed by Landsteiner haptens, which confer upon certain bacterial antigens their strict immunological specificity.

The addition of the purified polysaccharide to the homologous antibacterial serum removes by precipitation the whole of the precipitins engendered by the specific antigen associated with the smooth form of *B. dysenteriae* (Shiga). Furthermore, antibacterial sera that have been completely absorbed with the polysaccharide no longer give specific agglutination with suspensions of Shiga's bacillus nor do they yield precipitates when treated with simple acid, neutral or alkaline extracts or autolysates of the intact organism. These experimental results, when considered together, give strong support for the belief that the highly purified polysaccharide hapten accounts completely for the serological phenomena of specificity of *B. dysenteriae* (Shiga).

The specific substance of *B. dysenteriae* (Shiga) appears, therefore, to belong to a group of substances, the complex polysaccharides, which possess colloidal properties and high molecular weights and for which adequate criteria of homogeneity are difficult to prescribe and determine. It is impossible, therefore, to claim with certainty that the specific substance, in its present state of purity, is a single chemical compound. However, after each stage in its purification the polysaccharide preparation has shown a corresponding change in its chemical and physical properties together with an increase in serological activity as measured by specific precipitation with the homologous immune rabbit or horse serum and a diminution in the minimum amount of the polysaccharide substance necessary for the induction of a specific and fatal anaphylactic shock in guinea-pigs that have been sensitised with the homologous antibacterial serum from the rabbit [Morgan, 1932]. The purified specific polysaccharide, $[\alpha]_D + 98^\circ$, which has been described in this paper, has remained unchanged in properties following the application of several methods of purification. Thus, conversion of the specific polysaccharide into the acetyl or benzoyl derivative and its regeneration by treatment with alkali yields the specific substance unchanged in all the chemical and immunological properties examined. Furthermore, the prolonged action of nitrous acid in the presence of dilute mineral acid fails to liberate the nitrogen contained in the specific complex or to alter the immunological properties of the polysaccharide. Finally, the repeated fractionation of the specific substance from acetic acid solution or from aqueous or pyridine solution by alkaline, neutral or acid alcohol or acetone has also failed to yield a specific polysaccharide substance with different chemical and immunological properties. These facts when considered together with the other data presented suggest that the polysaccharide substance which possesses an optical rotation $[\alpha]_D + 98^\circ$ and a nitrogen content

of 1.61 %, is the specific bacterial polysaccharide hapten virtually freed from other non-specific polysaccharide impurities.

The properties of the specific polysaccharide, so far as they are known at present, support the belief that the basic structural unit in the polysaccharide aggregate consists of four hexose molecules and one *N*-acetylaminohexose molecule. The unhydrolysed polysaccharide gives an iodine reduction which is equivalent to 3.4 % of glucose and this reducing figure would be given by a polysaccharide which has one hexose end-unit, possessing a free aldehyde group, in each group of 30 of the hexoses that constitute the whole polysaccharide molecule. It would appear therefore that the basic hexose unit repeats itself six times in the polysaccharide molecule. The molecular weight of the polysaccharide calculated from the hypiodite titration is about 5100.

The polysaccharide (10 mg.) gives only a very faint test—representing less than 0.05 mg.—for the presence of *N*-acetylaminohexose, whereas if the terminal reducing group of the polysaccharide were associated with a *N*-acetylaminohexose molecule a quantitative determination would show the presence of about 3.5 % of *N*-acetylhexosamine. The reducing end-unit in the polysaccharide structure is therefore not a *N*-acetylaminohexose molecule. Acid hydrolysis of the polysaccharide under conditions that do not remove the acetyl groups from the *N*-acetylhexosamine molecules, rapidly breaks the glycosidic linkages which join the aldehyde groups of the *N*-acetylaminohexose molecules with their adjacent hexoses. It has been found that when, under these conditions of hydrolysis, the reducing power reaches about 45 % (H. and J.) and 30 % (hypiodite), the whole of the nitrogen in the polysaccharide can be estimated colorimetrically as *N*-acetylaminohexose, a result which indicates that each *N*-acetylhexosamine molecule now possesses a reducing aldehyde group.

When the specific substance is heated with dilute mineral acid the serological properties are rapidly destroyed and their destruction is complete when the reducing power of the solution has reached only about one-third of its final value. It would appear therefore that as soon as certain linkages within the polysaccharide molecule are broken and long before the whole of the component hexoses are liberated as single molecules the substance loses its power to precipitate with the homologous immune serum and to induce anaphylaxis in guinea-pigs that have been passively sensitised with an antibacterial *B. dysenteriae* (Shiga) rabbit serum.

The products of partial hydrolysis at this stage of the hydrolysis, however, still possess some degree of serological activity since when they are added in a dilution as high as 1 : 3000 to the immune serum they cause a specific inhibition of the precipitation which normally takes place on the subsequent addition of the appropriate quantity of the specific polysaccharide. Furthermore the partial hydrolysis products are also able to desensitise guinea-pigs which have been passively sensitised with immune serum and thus protect them against the anaphylactic shock that is induced by a subsequent intravenous injection of the intact polysaccharide. These experimental results suggest that the chemical structure which is responsible for the specificity of the polysaccharide remains intact after the whole of the *N*-acetylaminohexose in the polysaccharide has been liberated in the form of single molecules or, as appears more probable, as end-units in the oligosaccharides that are formed. Experiments designed to isolate from the hydrolysis products the chemical substance which has the power to inhibit the specific polysaccharide-antibody reaction are in progress.

During the course of this work several non-specific polysaccharide substances have been isolated from their association with the specific polysaccharide of

B. dysenteriae (Shiga). At least two of the non-specific polysaccharides react with immune sera which contain anti-agar bodies and these appear to be associated in some way with the agar upon which the organisms have been grown. The chemical and physical properties of these two serologically reactive but non-specific substances differ widely from the corresponding properties of agar, which indicates that they have been derived from the agar by enzymic processes which are most probably associated with the growth of the dysentery bacillus.

An examination of the literature reveals the frequency with which workers are content to designate their specific bacterial polysaccharide preparations "pure" provided that they are protein-free and essentially polysaccharide in nature, while the presence of non-specific polysaccharides and serologically reactive agar-like substances is frequently overlooked. It is suggested that serological tests for the presence of agar-like carbohydrate should be carried out on all specific polysaccharides that have been obtained from organisms grown on nutrient agar. Similarly, preparations of specific polysaccharide which have been derived from organisms that have been grown on solid (agar) medium or in broth cultures should be carefully examined for freedom from non-specific polysaccharides before they are accepted as "pure" for immuno-chemical studies.

SUMMARY.

1. A modified method is described for the isolation and purification of a specific polysaccharide from the "smooth" form of *B. dysenteriae* (Shiga).

2. The polysaccharide is strongly dextrorotatory, $[\alpha]_D + 98^\circ$, it contains 1.6% nitrogen the whole of which is present in the form of acetylated amino-groups, and it yields 97% of reducing sugars on acid hydrolysis.

3. The specific substance accounts completely for the serological specificity of *B. dysenteriae* (Shiga).

4. The occurrence and significance of certain non-specific polysaccharides is discussed.

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