CCXLVIII. STUDIES IN IMMUNO-CHEMISTRY

II. THE ISOLATION AND PROPERTIES OF A SPECIFIC ANTIGENIC SUBSTANCE FROM *B. DYSENTERIAE* (SHIGA)

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DURING the last two decades numerous investigators have attempted to isolate specific antigens from bacteria but in no instance have they been able to obtain such substances in a chemically pure form and in an immunologically active condition. Nevertheless the results of these researches, which were initiated by Dochez & Avery [1917], were of the greatest importance because later studies, which developed as a direct result of this work, have formed the basis for our present knowledge of antigenic structure. In continuation of the early observations of Perlzweig & Steffen [1923] and of Perlzweig & Keefer [1925], several workers, notably Schiemann & Casper [1927], Schiemann *et al.* [1931], Day [1928; 1930; 1933], Wadsworth & Brown [1931; 1933], Felton [1932] and Harley [1934; 1935], have described methods of isolating bacterial antigens which, according to some of these workers, were most probably polysaccharide in nature. The chemical evidence produced was, however, not strong enough to establish with certainty that the antigens obtained were protein-free. Indeed it was not until quite recently that Avery & Goebel [1933], Pappenheimer & Enders [1933] and Enders & Wu [1934] brought forward indisputable evidence that an acetylated polysaccharide which they had isolated from *Pneumococcus* (Type I) possessed antigenic properties. Thus mice inoculated intraperitoneally with very small quantities of the substance showed a type-specific immunity to subsequent infection with the virulent organism. These experiments revealed several points of difference between the antigenic properties of the acetylated polysaccharide and those of the intact virulent coccus. For example, it was not found possible to produce a type-specific agglutinin, precipitin or protective antibody when the rabbit was immunized with the acetyl-polysaccharide whereas these immune bodies are readily produced in this animal by a suspension of the organism. It seemed clear therefore that the complete type-specific antigen still remained to be isolated and that although the acetylated polysaccharide was an important constituent of the antigenic complex it did not possess the full antigenicity of the naturally occurring antigen. Similar results have been described by Topley [1937] for the polysaccharide component of the antigen of *B. aertrycke*.

Recently two independent researches have been published which demonstrate beyond question the non-protein nature of certain bacterial antigens. Boivin and his co-workers [Boivin & Mesrobian, 1933; Boivin *et al.* 1935; Mesrobian, 1936] have isolated the specific antigen from several species of bacteria by extracting them for a short time with ice-cold trichloroacetic acid (pH 1.0–2.0). Raistrick & Topley [1934] and Topley *et al.* [1937] have also

obtained bacterial antigens by a modification of the method originally used by Douglas & Fleming [1921] of digesting the acetone-killed organisms with trypsin at pH 8.5 for 5 days at 37°. So far as is known, neither of these methods of isolation alters in any way the immunological properties of the particular antigens that have been examined, but instances are already known where antigenic components have been damaged by treatment with a too strong acid or alkali and it seems improbable, therefore, that the immunological properties of certain labile antigens will remain unmodified when they are exposed for any appreciable length of time to degrees of acidity or alkalinity which are far removed from neutrality.

The risk of altering the chemical or immunological properties of the antigenic complex by too drastic chemical manipulation was considered and a method for isolating antigens which depends on the solubility of the complexes at a neutral reaction and at normal or low temperatures in simple organic solvents has been evolved. The technique together with some observations on the chemical and immunological properties of the antigenic complex of *B. dysenteriae* (Shiga) are given in this communication.

EXPERIMENTAL

The extraction method for isolating the antigenic constituent of *B. dysenteriae* (Shiga) originated from an observation that the specific polysaccharide of this organism showed a definite solubility in glycerol, ethyleneglycol, diethyleneglycol, propyleneglycol, 2:3-butyleneglycol, ethylenechlorohydrin and trimethyleneglycol. Further experiments showed that other specific and also non-specific polysaccharides were soluble in these organic solvents. Indeed the solubility of the specific polysaccharide of *B. dysenteriae* (Shiga) in ethyleneglycol and its subsequent fractional precipitation from solution with alcohol was used as early as 1933 to determine its homogeneity. Somewhat later the solubility of a number of dry protein preparations in these organic solvents was also examined; they were found to be relatively insoluble. Table I gives the results of experiments in which the solubilities, under similar conditions, of a number of polysaccharides and proteins in the various organic solvents were roughly determined. No attempt has been made to ascertain the exact solubilities of these substances and the results are merely expressed as (1) completely soluble (C), (2) partly soluble (S), and (3) insoluble (O). It will be seen that complete solubility represents a concentration of approximately 1% and it is at once apparent that there is a sharp difference in the solubilities of these two classes of substance. The solubilities of some of the polysaccharides were also determined for the solvents, monoacetin, diacetin, the monomethyl ether of ethyleneglycol and ethyleneglycol monoacetate, but most of them were found to be insoluble or of such low solubility as to render the method of no practical importance.

In the light of previous work, which had shown that the specific polysaccharide component of numerous pathogenic micro-organisms accounted almost completely for the serological specificity of the organism and was therefore presumably an important and major constituent of the complete bacterial antigen, the solubilities of the bacterial polysaccharides in ethyleneglycol, diethyleneglycol, trimethyleneglycol and the other solvents suggested that the antigens might also be soluble to some extent in one or other of these compounds; some simple experiments showed the correctness of this assumption. A preliminary account of the isolation of the antigen of *B. dysenteriae* (Shiga) by this method has already been given [Morgan, 1937].

Table I. *Showing the solubilities of some polysaccharide and protein substances in various organic solvents*

Substances 50 mg.	Solvent (5.0 ml.)				
	Ethylene- glycol	Diethylene- glycol	Glycerol	Trimethyl- leneglycol	Mono- methyl ether of ethylene- glycol
Polysaccharides:					
Glycogen	C	S	S	O	O
Inulin	C	C	C	C	O
Mannan	C	S	?	—	O
Gum arabic	C	O	O	—	O
Yeast gum	C	S	C	—	—
Galacto-carolose*	S	S	C	—	—
Manno-carolose*	C	S	C	—	—
Chondrosine	C	C	C	C	C
Bacterial polysaccharides from:					
<i>B. dysenteriae</i> (Shiga)	C	C	C	C	O
<i>Pneumococcus</i> (Type I)	S	O	Trace	—	O
<i>Pneumococcus</i> (Type II)	C	S	S	—	O
<i>Meningococcus</i> (Types I-III)	S	S	C	—	—
<i>B. proteus</i> (O. X 19)	S	S	S	S	O
<i>B. suispestifer</i>	C	S	S	S	—
Protein:					
Horse serum protein	O	O	O	O	O
Edestin	O	O	O	O	O
Egg albumin	O	O	O	O	O
Casein	S	O	O	O	O

C, completely soluble; S, partly soluble; O, insoluble.

* The author is indebted to Prof. H. Raistrick for samples of these substances.

The extraction of a specific antigenic substance from the "smooth" form of B. dysenteriae (Shiga)

The bacteria (strain K 624) were grown for 18 hr. at 37° on nutrient agar in covered metal dishes measuring 15 × 11 in. The growth was scraped off and re-suspended in a small volume of ice-cold saline. The bacteria were immediately washed once with saline, three times with cold acetone and rapidly dried *in vacuo*. A typical extraction experiment was carried out as follows. The dry bacteria were weighed (50 g.) into a litre bottle and 500 ml. of diethyleneglycol at 37° were added with vigorous shaking. A sample (0.5 ml.) of the suspension was removed, diluted with 4.5 ml. of water and the pH determined; this should be between 6.5 and 7.0. After standing for 2 hr. at 37° the suspension was mechanically shaken for an hour and then allowed to remain at 0° or at 15–18° for 24 hr. During this period the suspension was frequently shaken by hand and finally given an hour's vigorous mechanical shaking and kept for a further period of 24 hr. At the end of this time the bacterial suspension was centrifuged for $\frac{1}{2}$ hr. at 35,000 r.p.m. in a closed bowl of a Sharples supercentrifuge. The clear, slightly opalescent supernatant fluid was finally clarified from any residual bacteria by filtration through a British Berkefeld filter candle. A faintly opalescent solution of the antigen in the organic solvent was thus obtained. The antigenic material was obtained in aqueous solution by dialysis at 0°; the organic solvent rapidly diffused through the cellophane membrane, which was found to be completely impermeable to the bacterial substance. During the dialysing process the antigenic substance passes from a state of true solution in the organic solvent to one of colloidal suspension in water and the final preparation after dialysis is strongly opalescent and shows, on agitation, a shimmer

characteristic of rapidly growing bacterial suspensions or suspensions of certain crystalline protein preparations. Colloidal solutions of the antigen prepared in this manner gave no deposit when centrifuged at 4000 r.p.m. in an angle centrifuge. An aqueous solution of the antigenic material could be obtained at any desired concentration by evaporation *in vacuo* at low temperatures, and rapid evaporation from the frozen state has been found to produce a fine, white sponge-like mass which will again readily pass into colloidal solution on the addition of water or saline. Solutions of the antigenic material are stored at 0° in the presence of toluene.

The first treatment of the bacteria with the organic solvent does not remove the whole of the bacterial antigen from the micro-organisms and it is considered advisable to perform at least two further extractions in order to obtain a reasonable yield. The exact conditions for the isolation of the antigen, and the most suitable solvents to employ, must be determined for each kind of micro-organism investigated.

A further purification of the antigenic substance has been carried out by the fractional precipitation of the aqueous solution of the active material by acetone or alcohol. A 0.5–1 % solution of the antigen does not precipitate until the concentration of acetone reaches at least 50 % by volume and precipitation of the greater part of the antigen is complete when the acetone concentration reaches 66 %. The fractionation process should be carried out at 0° and the precipitated antigen re-suspended in water at this temperature and finally the antigenic preparation obtained free from traces of acetone by dialysis against ice-cold distilled water. The further addition of acetone to the 66 % acetone supernatant fluid up to 75 % by volume produces a small precipitate which on re-solution in water gives rise to a clear colourless solution which in no way resembles the thick milky opalescence which is characteristic of the first acetone fraction (50–66 %) when it is re-dispersed in water. The final fraction, which is soluble in 75 % acetone, can be obtained in the form of an opalescent solution if it is concentrated to a small volume *in vacuo*. A 1 % solution of the antigenic material is not completely precipitated until 4 vol. of ethyl alcohol have been added, although cooling to 0° and the addition of a trace of potassium acetate will frequently cause a small precipitate to separate with the addition of 3 vol. of alcohol.

The large number of animals required to obtain even an approximate idea of the relative antigenic powers of the various fractions has precluded extensive immunological tests. However, the experimental results that have been obtained indicate clearly that the first acetone precipitate (50–66 %) and the main alcohol precipitate (75–80 % by volume) contain the greater part of the antigenic material. Other methods of separation that involve the fractional precipitation of the antigenic material from its solution in organic solvents are being examined and attempts are being made to effect the removal of non-specific material by a process which utilizes adsorption and elution of the active substance.

An examination of the corresponding "rough" variant (strain K 624) of *B. dysenteriae* (Shiga) has been made and in two experiments 30.5 and 23 g. of the dry organisms were extracted with ethyleneglycol and diethyleneglycol respectively. The total amount of soluble material was in each case less than 1 % of the weight of the dry bacillary mass and the final preparations, after removing a heavy gelatinous precipitate that formed during dialysis, were found to give aqueous solutions that were almost entirely free from opalescence and were practically non-antigenic. The portion of the extracted material that was soluble in water contained only 1.4 % nitrogen and 0.13 % of phosphorus.

The chemical properties of the antigenic material

The most active preparations even in a concentration as low as 0.1% yield opalescent solutions in water or saline. For this reason it is somewhat difficult to ascertain whether a slight precipitate is produced or not on the addition of a chemical reagent in qualitative tests. The reagents were added to a solution of the antigen preparation and after the mixtures had stood overnight at 0° they were centrifuged at low speed and the formation of a precipitate recorded. In this way the following results were obtained. The addition of a solution of lead acetate, copper sulphate, mercuric acetate, phosphotungstic acid (with and without sulphuric acid) or uranium acetate to the active material caused no precipitation. Salicylsulphonic acid, trichloroacetic acid and picric acid (Esbach's reagent) tests for protein were negative when applied to a 1.0% solution of the antigenic material. The ninhydrin test was negative but the biuret test was positive. A preparation of the antigenic material that had been treated with 0.5*N* trichloroacetic acid at 0° for 2 hr., according to the technique described by Boivin *et al.* [1935], reacted in all the qualitative tests here described in a similar manner to the antigenic substance extracted by means of diethylene-glycol. The two preparations gave positive biuret tests of approximately equal intensity and colour when 20 mg. of the material were employed in a volume of 2 ml. The naphthoresorcinol test for glucuronic acid, the orcinol and phloroglucinol tests for pentoses and Selivanoff's reaction for ketoses were consistently negative on all the samples examined. The material gave an immediate yellowish red colour when treated with a sodium carbonate solution of diazobenzene-sulphonic acid. The iodine test for glycogen was negative.

After precipitation from aqueous solution at 0° by alcohol or acetone the antigenic material is obtained as a white amorphous substance. A typical analytical result obtained for the substance after drying at 40° over phosphorus pentoxide *in vacuo* was: C, 45.5; H, 7.6; N, 3.8 and P, 1.3%, but the figures for N and P varied somewhat for different preparations. A preparation after treatment with trichloroacetic acid gave the following results: C, 45.4; H, 7.6; N, 3.7 and P, 1.3%. None of the phosphorus was in the form of inorganic phosphate. The various preparations usually contained 4–6% of non-combustible residue.

The antigenic material (1.43 g.) was hydrolysed by dilute (0.1*N*) acetic acid (200 ml.) at 100°. During the hydrolysis the opalescence of the solution disappeared and a white precipitate was formed but at this stage of the hydrolysis the solution was found to possess practically no reducing power as measured by the method of Hagedorn and Jensen. A positive Ehrlich reaction for *N*-acetylhexosamine was not given by the solution. The heating was continued for $\frac{1}{2}$ hr. after the opalescence had disappeared and the solution was then cooled and left for several hours at 0°. The insoluble precipitate was removed, washed three times with cold water and the solution together with the washings were concentrated *in vacuo* to 10–20 ml. The concentrated solution was subsequently dialysed to remove salts and poured into several volumes of alcohol at 0°. After two precipitations from aqueous solution by alcohol or acetone the material that was obtained was identical, in the properties that were examined, with the specific polysaccharide that was isolated [Morgan, 1936] by other means from the intact "Shiga" dysentery bacillus. The Molisch reaction was given by solutions of the substance containing only 1 part in 500,000. The material isolated in this manner from the antigen gave a clear solution in water, was practically non-reducing, H. and J. 3.2%, and was strongly dextrorotatory,

$[\alpha]_D + 98^\circ$. It contained 1.8 % nitrogen; phosphorus and sulphur were absent. The material reacted with anti-bacterial *B. dysenteriae* (Shiga) immune serum at a dilution of 1 : 12,000,000, but gave no visible precipitation at any dilution from 1 : 1000 to 1 : 10,000,000 with an anti-agar immune serum. The polysaccharide weighed 0.68 g. after dialysis and precipitation with alcohol and represents 47.5 % by weight of the antigen preparation. In another similar experiment the polysaccharide component formed 48.5 % of the total antigenic complex. Mesrobian & Calalb [1936] found a slightly lower percentage for the polysaccharide present in the antigenic material they had isolated by means of the trichloroacetic acid extraction process.

The insoluble precipitate that separated during the hydrolysis of the substance was collected by centrifuging, dissolved in weak sodium hydroxide, diluted to 250 ml. and reprecipitated by the addition of 0.1 *N* acetic acid; the process of solution and precipitation was repeated three times and the material was dried *in vacuo*. The substance weighed 0.386 g. and represented 27 % by weight of the antigenic complex hydrolysed. Another preparation gave 26 % of similar material. The substance gave a strong biuret test and ninhydrin reaction and the addition of diazobenzenesulphonic acid in sodium carbonate solution to an alkaline solution of this substance gave an immediate brick-red coloration. The Millon reaction was complicated owing to the precipitation of the substance by the acid reagents employed. Nevertheless it appeared to be a positive reaction since the precipitated material developed a reddish coloration after a few minutes' heating at 100° with the Millon reagent. Analysis: N, 7.1; P, 1.5 %. Reducing power as measured by the Hagedorn and Jensen method 8.5 %.

A portion of the insoluble material (0.200 g.) was thoroughly extracted with dry methyl alcohol and the combined alcoholic supernatant fluids were filtered and evaporated to dryness *in vacuo*. A white, waxy solid was obtained which weighed 74 mg. and represented approximately 10 % of the antigenic complex. The whole of the extracted material dissolved in dry ether and, on subsequent evaporation and treatment with 50 % aqueous methyl alcohol, required 15.4 ml. of 0.01 *N* sodium hydroxide for solution and neutralization to a pink coloration with phenolphthalein.

After removing the methyl alcohol- and ether-soluble material from the insoluble acid hydrolysis residue there remained 0.126 g. of a white solid which appeared to be insoluble in water but dissolved readily in dilute sodium hydroxide solution. The fraction represented 17 % of the antigenic substance; it contained 10.2 % N, 1.1 % P and possessed a specific rotation, $[\alpha]_D - 54^\circ$. The material gave an intense biuret reaction, but the ninhydrin test was rendered doubtful owing to the insolubility of the fraction in water or weakly acid solution and the inhibition caused by the alkaline reaction when material dissolved in NaOH was employed. The addition of diazobenzenesulphonic acid to an alkaline solution of the substance gave rise to an immediate and intense red coloration.

The specific polysaccharide and the insoluble substance derived from the antigenic complex as a result of acid hydrolysis together weighed 1.06 g. and represented 74 % of the antigen preparation. This figure indicated that 28 % of the complex had escaped isolation and subsequently 0.198 g. (14 %) of the substance was recovered from the distilled water employed for the dialysis of the polysaccharide fraction. The diffusate consisted of a mixture of substances and contained about 2.0 % N and 2.0 % of total P of which about half was present as inorganic phosphate. The antigenic material before hydrolysis contained phosphorus in organic combination only; therefore, during the hydrolytic process a part of the phosphorus had been liberated. This fraction gave negative

biuret, ninhydrin and Millon reactions. The addition of diazobenzenesulphonic acid in sodium carbonate solution to the diffusate gave an immediate reddish yellow coloration. The fraction possessed a reducing power (H. and J.) of 7.8% and a specific rotation, +39°, calculated on the total dry weight.

A detailed study of the hydrolysis products of the antigenic complex will be undertaken when larger quantities of the material are available.

IMMUNITY EXPERIMENTS

The subcutaneous, intravenous or intraperitoneal injection of living *B. dysenteriae* (Shiga) into the rabbit results in death of the animal from toxæmia and the course of the illness can in no way be considered to be comparable with a severe dysentery attack in man. Indeed, all attempts to produce an experimental infection in the rabbit have failed. This generally accepted fact makes it impossible to immunize rabbits with the various antigenic preparations and to test the degree of the resulting immunity by subsequently infecting the animal with the homologous living organism. We have been forced, therefore, throughout this work, to measure the immune response by *in vitro* reactions and to express the results in terms of agglutination or precipitation titre or as mg. of antibody protein contained in 1 ml. of the immune serum. The quantitative precipitin technique as elaborated by Heidelberger *et al.* [1933] has been employed for this purpose.

The antigenicity of the various preparations and fractions obtained from *B. dysenteriae* (Shiga) was roughly evaluated by giving to groups of rabbits three intravenous doses, each of 0.05 mg., at 3–4 day intervals; the animals were bled before the immunization and 1 week after the last dose of antigen. The results of the preliminary antigenicity tests which were made on all the extracts, irrespective of the nature of the solvent or of the length of the extraction period, will not be given, because in every case the response was only slightly lower than that shown by the first example given in Table II which illustrates the antigenic response from a course of 6 doses of a crude bacterial extract that had been obtained by means of ethyleneglycol. It will be seen that the serum of each of the three rabbits (Nos. 361, 362 and 363) which had been immunized reached an agglutination titre of 1 : 1000, 1 : 2000 and 1 : 5000 respectively. The influence of the size of the immunizing dose and of the interval between the doses has not been considered and it may well be that under optimum conditions the ultimate response would have been even greater. Slightly better results, as measured by the simple agglutination technique, were obtained when only 3 doses of a fraction of the antigenic material, which had been precipitated with acetone between 50 and 62%, were employed (rabbits Nos. 376, 377 and 378). Subsequently a fraction which had been obtained within even narrower limits (55 and 62% acetone) was found to be somewhat more active when it was tested in a group of three rabbits (Nos. 385, 386 and 387) and it was therefore retested using only 0.005 mg. for each of the immunizing doses. The results (rabbits Nos. 388 and 389, Table II) show that an agglutination titre of 1 : 5000 was reached in the serum of both animals. Similar immunity experiments which employed material that had been fractionated by alcohol gave almost identical results; one example is given in Table II (rabbits Nos. 380 and 381). Immunization with the whole ethyleneglycol extract from the “rough” strain of *B. dysenteriae* (Shiga) failed to produce any appreciable immune response. Two rabbits, Nos. 383 and 384, that received 6 doses of 0.05 mg. of this preparation showed only a trace of agglutination at 1 : 50 and 1 : 100 respectively. Normal

Table II. *Showing the agglutination titres of rabbit and horse immune serum prepared against antigenic fractions isolated from B. dysenteriae (Shiga)*

Rabbit no.	mg. per dose	No. of doses	Nature of material	Dilution of serum giving agglutination										
				1:10	1:20	1:50	1:100	1:200	1:500	1:1000	1:2000	1:5000		
361	0.05	6	Whole ethyleneglycol extract	{ 4	4	4	4	3	2	1	0	0		
362				{ 4	4	4	4	4	4	2	1	0		
362				{ 4	4	4	4	3	1	0	0	0		
376	0.05	3	50-62% acetone fraction from diethyleneglycol extract	{ 4	4	4	4	4	4	3	2	0		
377				{ 4	4	4	4	4	4	4	3	0		
378				{ 4	4	4	4	4	4	3	1	0		
385	0.05	3	55-62% acetone fraction from diethyleneglycol extract	{ 4	4	4	4	4	4	3	2	1		
386				{ 4	4	4	4	4	4	3	2	1		
387				{ 4	4	4	4	4	4	3	3	1		
388	0.005	3	"	{ 4	4	4	4	4	4	3	2	1		
389				{ 4	4	4	4	4	4	3	3	1		
389				{ 4	4	4	4	4	4	4	3	1		
380	0.05	3	75-80% ethyl alcohol fraction from diethyleneglycol extract	{ 4	4	4	4	4	4	3	2	0		
381				{ 4	4	4	4	4	4	3	2	1		
381				{ 4	4	4	4	4	4	3	2	1		
383	0.05	6	Whole extract of "rough" <i>B. dysenteriae</i> (Shiga)	{ 4	2	1	0	0	0	0	0	0		
384				{ 4	3	2	1	0	0	0	0	0		
384				{ 4	3	2	1	0	0	0	0	0		
Horse 1	2.7	6	75-80% ethyl alcohol fraction from diethyleneglycol extract	4	4	4	4	4	4	4	4	2		
Horse 2	2.7	3	"	4	4	3	1	0	0	0	0	0		

4, complete agglutination (or haemolysis).
 3, almost complete agglutination (or haemolysis).
 2, incomplete agglutination (or haemolysis).
 1, trace of agglutination (or haemolysis).
 0, no agglutination (or haemolysis).

rabbit serum seldom shows homologous agglutination at a dilution greater than 1 : 10.

A quantitative determination of the amount of antibody protein has been carried out on a number of the immune rabbit sera and one immune horse serum. The following method has been employed. The serum sample, 5 ml. was treated with a 1 : 10,000 dilution of the specific polysaccharide (N, 1.7 %; $[\alpha]_D + 98^\circ$) of *B. dysenteriae* (Shiga) until the whole of the antibody was precipitated. The precipitate was thoroughly washed with ice-cold saline, dissolved in dilute sodium hydroxide and the total nitrogen estimated. Similar experiments were made using a 1 : 10,000 dilution of the homologous antigen preparation in place of the specific polysaccharide. Each of the determinations was made in duplicate. The results obtained with five different rabbit sera are shown in Table III. The rabbits received 6 doses of 0.050 mg. of the antigen intravenously at 3-4 day intervals and were bled 1 week after the last dose of antigen.

Table III. *Showing the amounts of protein nitrogen precipitated from an immune serum by (a) the specific polysaccharide and (b) the homologous antigen preparation*

Rabbit no.	mg. of protein nitrogen precipitated from 1 ml. immune-serum by		Difference mg. nitrogen
	(a) 1 : 10,000 solution of specific polysaccharide	(b) 1 : 10,000 solution of the homologous antigen	
508	0.131	0.120	-0.011
509	0.128	0.109	-0.019
510	0.054	0.054	0.00
512	0.105	0.098	-0.007
513	0.240	0.260	+0.020

It will be observed that in three of the determinations somewhat less antibody protein was precipitated by the homologous antigen preparation than by specific polysaccharide, whereas in one instance the antigen precipitated more protein from the immune serum than was precipitated by the specific polysaccharide. These experimental results are difficult to interpret since in each sample duplicate estimations of the nitrogen in the protein precipitated agree to within 3 %. Furthermore, all the precipitation experiments and the subsequent washing of the precipitated protein were carried out at one time in a thermostatically controlled cold room (0-2°). It was noted, however, that the end-point of precipitation in those experiments in which the specific polysaccharide was employed was extremely sharp, whereas it was somewhat difficult to determine the exact end-point of precipitation when the antigen solution was employed. The precipitate produced on the addition of the specific polysaccharide to the immune serum rapidly flocculated and settled to the bottom of the tube leaving a clear supernatant fluid. The addition of the antigen preparation caused a clouding of the serum with subsequent slow formation of rather loose, fine flocculi. It is therefore conceivable that the low values for the protein precipitated by the antigen may be due to the difficulty of determining the exact end-point of the antigen-antibody reaction. It may be that the antigen-antibody complex has a slight but appreciable solubility in saline at 0° and therefore when it is exposed to the same washing technique as is usually applied to polysaccharide-antibody precipitates a portion of the complex dissolves. Further quantitative determinations of immune body are being carried out with other serum samples in order to ascertain a reason for the low precipitation values that have been obtained.

Two horses were immunized intravenously with a preparation of the antigenic material which had been obtained by alcohol precipitation between the limits 75 and 80 %. After 3 doses, each consisting of 2.7 mg. of the material dissolved in 20 ml. of saline, serum samples taken 1 week after the last dose agglutinated the homologous organism to a dilution of 1:500 and 1:100 respectively. Three further doses given to one of the horses produced a serum which agglutinated "Shiga" bacilli at a dilution of 1:5000. A quantitative experiment to determine the amount of antibody protein per ml. of the immune serum was performed on the serum of one horse. Two 5 ml. samples of the serum were separately treated in exactly the same manner as has already been described for the immune rabbit sera with (1) a 1:10,000 solution of the homologous specific polysaccharide, and (2) a 1:10,000 solution of the antigenic material until precipitation was complete. The amount of nitrogen per ml. was determined and the total protein precipitable by the polysaccharide and the antigenic material was calculated. The polysaccharide precipitated 1.47 mg. and the antigenic material 1.64 mg. of protein from each ml. of the immune horse serum. During the experiments with both rabbit and horse immune-sera it was observed that the volume of 1:10,000 polysaccharide solution required to precipitate the antibody present was always about 40 % of the volume of 1:10,000 antigen solution necessary to accomplish the same process. These facts suggest that it is only the polysaccharide portion of the antigenic complex that binds the antibody molecule and that this portion of the antigen alone is the one that is primarily responsible for the formation of the insoluble antigen-antibody complex.

The production of "Shiga" heterophile antibody

The examination of the immune sera produced by various fractions of the antigen preparation revealed the presence of "Shiga" heterophile immune body in nearly all of the specimens examined. This result was somewhat surprising for it is well known that only a proportion of rabbits immunized with *B. dysenteriae* (Shiga) yield haemolytic antibody. The results of the tests for heterophile immune substance are given in Table IV. In all cases a normal bleeding was taken from each rabbit before the course of immunization and a sample of this serum was included in each test for heterophile antibody. Normal sera have generally been found to give only a trace of haemolysis at a dilution of 1:50 under the experimental conditions employed.

The serum of rabbits that have been immunized with *B. dysenteriae* (Shiga) and have produced "Shiga" heterophile immune body frequently shows a rapid fall in the amount of this immune substance during the course of subsequent doses of the bacterial antigen. Table IV shows that this decrease also occurs during immunization with the antigenic fractions. For example, the serum of rabbit 361, when diluted 1:2000 with saline, gave rise after 3 doses of the antigen to definite haemolysis of sheep red cells suspensions in the presence of complement. After the sixth dose of antigen it was necessary to employ nearly ten times as much serum to obtain the same degree of haemolysis, and finally, after 9 doses of the antigen, the serum failed to cause haemolysis of red cells when diluted more than 1:100 with saline. A more detailed study of this phenomenon is being carried out.

In view of the regularity with which the haemolytic immune body was produced it was considered advisable to investigate the possibility of the immune substance being formed as a result of non-specific stimulation. The following neutralization and adsorption tests were therefore performed and the serum

Table IV. Showing the production of "Shiga" heterophile immune body in rabbits inoculated with fractions isolated from *B. dysenteriae* (Shiga)

Rabbit no.	Nature of material	mg. per dose	No. of doses	Dilution of serum giving haemolysis							
				1:50	1:100	1:200	1:500	1:1000	1:2000	1:5000	
361	Whole ethyleneglycol extract	0.05	3	{ 4	4	4	3	2	1	0	
362				{ 4	4	3	2	1	0		
363				{ 4	4	3	2	1	0		
361	"	0.05	6	{ 4	3	2	0	0	0	0	
362				{ 4	2	1	0	0	0		
363				{ 2	1	0	0	0	0		
361	"	0.05	9	{ 4	2	0	0	0	0	0	
362				{ 4	2	0	0	0	0		
363				{ 1	0	0	0	0	0		
376	50-62% acetone fraction from diethyleneglycol extract	0.05	3	{ 4	4	4	3	2	1	0	
377				{ 4	2	1	0	0	0		
378				{ 4	4	4	4	4	4	1	
380	75-80% alcohol fraction from diethyleneglycol extract	0.05	3	{ 4	4	4	4	3	1	0	
381				{ 4	3	1	0	0	0		
385	55-62% acetone fraction from diethyleneglycol extract	0.05	3	{ 4	3	2	1	0	0	0	
386				{ 4	4	3	2	1	0		
387				{ 4	4	4	4	2	1	0	
388	"	0.005	3	{ 4	4	4	4	3	1	0	
389				{ 4	2	2	0	0	0		

from rabbit No. 378 was used for this purpose. Seven 1.5 ml. portions of the serum diluted 1 : 25 were separately treated with (a) 1.5 ml. of a 1 : 1000 dilution of the specific "Shiga" polysaccharide, (b) 1.5 ml. of a 1 : 1000 dilution of the homologous antigen fraction, (c) a suspension of washed *B. dysenteriae* (Shiga), (d) a suspension of *B. dysenteriae* (Flexner Z), (e) a suspension of diphtheria bacilli and (f) a suspension of washed sheep red cells. The mixtures were kept at 37° for 2 hr. and overnight at 0° and were finally centrifuged at this temperature. A series of dilutions of each of the clear supernatants was tested for haemolytic antibody in the usual manner using sheep red cells and complement. The results, together with those obtained from experiments in which the corresponding normal serum was employed are shown in Table V. It

Table V. *Showing the neutralization or adsorption of the heterophile immune serum by (1) "Shiga" specific polysaccharide, (2) "Shiga" antigen and (3) B. dysenteriae (Shiga)*

Serum treated with	Dilution of immune-serum giving haemolysis						Dilution of normal serum giving haemolysis		
	1 : 50	1 : 100	1 : 200	1 : 500	1 : 1000	1 : 2000	1 : 50	1 : 100	1 : 200
"Shiga" polysaccharide	2	1	0	0	0	0	2	1	0
Antigenic fraction	1	0	0	0	0	0	1	1	0
"Shiga" bacilli	2	0	0	0	0	0	2	1	0
<i>B. dysenteriae</i> (Flexner Z)	4	4	4	4	3	1	1	0	0
Diphtheria bacilli	4	4	4	4	3	1	2	1	0
Sheep red cells	0	0	0	0	0	0	0	0	0
Unabsorbed (control)	4	4	4	4	4	2	2	1	0

will be seen that the heterophile immune body was completely neutralized by the specific "Shiga" polysaccharide and by the homologous antigen preparation and was completely adsorbed by the intact "Shiga" bacilli, whereas the non-specific micro-organisms failed to remove any appreciable quantity of the heterophile immune substance. These results indicate that the heterophile immune body produced is specifically related to a somatic antigen associated with *B. dysenteriae* (Shiga).

The action of acid and alkali on the antigenic material

(1) *The influence on agglutinin production.* Samples of the antigen preparation were adjusted to pH 4.2-4.4 by addition of an appropriate buffer solution and were heated at 100° for different periods of time in small sealed glass containers. The samples after heating were neutralized and diluted with saline to give solutions which contained 0.050 mg. of the antigenic material in a dose of 0.5 ml. Three rabbits (Nos. 385, 386 and 387) were given the unheated material while two rabbits were immunized with each specimen of the heated substance. Thus rabbits Nos. 390 and 391 received material that had been heated for 30 min., rabbits Nos. 392 and 393 received the substance after 60 min. heating and finally rabbits Nos. 394 and 395 received the antigen after it had been heated for 150 min. A precipitate formed in the solution after it had been heated for 30 min., but the supernatant fluid still remained slightly opalescent. Precipitation was observed to be complete after 1 hr. heating and the supernatant solution was perfectly clear. In each immunization a dose of 0.05 mg. of the heated antigen was injected at 3-4 day intervals on three occasions; serum samples were taken 1 week after the last dose of antigen had been given. The results of the agglutination tests carried out on the various serum samples are shown in Table VI. The antigenicity, as measured by

Table VI. *Showing the effect produced by acid and alkali at 100° on the immunological properties of the antigenic material*

Rabbit no.	pH	Time min.	Agglutination tests										Haemolysis tests									
			Dilution of serum giving agglutination												Dilution of serum giving haemolysis							
			1:10	1:20	1:50	1:100	1:200	1:500	1:1000	1:2000	1:5000		1:50	1:100	1:200	1:500	1:1000	1:2000				
385	—	—	{ 4	4	4	4	4	4	3	2	1		4	4	3	1	0	0				
386			{ 4	4	4	4	4	4	3	2	1		4	4	3	3	1	0				
387			{ 4	4	4	4	4	4	3	3	1		4	4	4	4	1	1				
390	4.2	30	{ 4	4	4	4	4	3	1	0	0		3	2	1	0	0	0				
391			{ 4	4	4	4	4	3	2	1	0		3	2	1	0	0	0				
392	4.2	60	{ 4	4	4	3	1	0	0	0	0		2	1	0	0	0	0				
393			{ 4	4	3	3	1	1	0	0	0		1	0	0	0	0	0				
394	4.2	150	{ 3	1	1	0	0	0	0	0	0		1	0	0	0	0	0				
395			{ 4	3	3	1	0	0	0	0	0		2	?1	0	0	0	0				
398	8.2	30	{ 4	4	4	4	4	3	2	0	0		3	2	1	0	0	0				
399			{ 4	4	4	4	3	2	1	0	0		4	4	2	0	0	0				
400	8.2	90	{ 4	4	4	3	3	2	0	0	0		2	1	0	0	0	0				
401			{ 4	4	4	4	3	2	0	0	0		2	1	0	0	0	0				
402	7.0	60	{ 4	4	4	3	2	1	0	0	0		1	0	0	0	0	0				
403			{ 4	4	4	3	2	0	0	0	0		1	0	0	0	0	0				

agglutinin response, is rapidly destroyed by heat and after 150 min. at pH 4.2–4.4 and 100° one of the two samples of immune serum which was examined failed to agglutinate *B. dysenteriae* (Shiga) at a dilution greater than 1 : 20; the other showed a titre of 1 : 100.

Similar treatment of the antigenic material at pH 8.2–8.4 was tested. After 30 min. heating, the material produced a high titre of agglutinins in the serum of the two rabbits, Nos. 398 and 399, which had been immunized, whereas even after 90 min. at 100° the titre of the two specimens of immune serum taken from rabbits Nos. 400 and 401 was in each case 1 : 500. It thus appears that the antigen is not rapidly destroyed when it is heated at 100° at pH 8.2–8.4.

The action of heat (100°) on an aqueous solution of the antigenic material at pH 7.0 was also determined and it was found that after 30 min. heating a precipitate formed and the opalescence of the solution disappeared. However, the preparation still possessed some antigenic action because the two rabbits which were immunized with the heated material produced specific agglutinins (rabbits Nos. 402 and 403) to titres of 1 : 500 and 1 : 200 respectively.

The influence on the immunizing potency of keeping the antigenic material at pH 6.0 and at pH 8.6 for 5 days at 37° was also determined. So far as could be ascertained incubation at pH 6.0 for 5 days caused no destruction of the antigen because the serum of each of two rabbits immunized with this material completely agglutinated the homologous organism up to a dilution of 1 : 2000. The serum samples that were obtained from two rabbits which had been given the alkaline antigen (pH 8.6) likewise possessed a high titre—1 : 5000 and 1 : 2000 respectively—of “Shiga” agglutinins.

(2) *The influence on the production of heterophile antibody.* The sera obtained in the immunity experiments designed to determine the action of acid (pH 4.2–4.4) and alkali (pH 8.2–8.4) at 100° were examined for their content of heterophile immune substance. The immune response with the normal, unheated antigen (rabbits Nos. 385, 386 and 387) is shown in Table VI. The power of the antigen to produce heterophile antibody is rapidly destroyed in acid solution at 100° so that after 60 min. heating the antigenicity of the preparation is negligible. Heating the antigenic substance at pH 8.2–8.4 likewise rapidly destroys its capacity to engender heterophile immune body. The two rabbits immunized with the antigenic preparation after it had been heated at pH 7.0 for 1 hr. both failed to produce heterophile antibody. Whether this lack of capacity to produce heterophile immune substance is due to a true destruction of the essential chemical structures in the antigen or is a direct result of the destruction of the colloidal nature of the antigen preparation has not been determined.

The sera of two rabbits which were immunized with an antigen preparation that had been kept at pH 6.0 and at 37° for 5 days contained a considerable quantity of “Shiga” heterophile antibody and they produced haemolysis at dilutions of 1 : 2000 and 1 : 500 respectively, whereas the immune sera of two rabbits which had received a preparation of the antigenic material that had been kept at pH 8.5 and at 37° for 5 days contained practically no “Shiga” haemolysins; both sera produced only a trace of haemolysis at a dilution of 1:100. These simple experiments indicate that the capacity of the antigen to produce heterophile immune body is easily destroyed by treatment with very weak alkali.

Action of enzymes on the antigenic material

Some preliminary experiments on the action of enzymes on the immunological properties of the antigenic material have been completed. An active preparation of trypsin was allowed to act at pH 8.5 for several hours on a purified preparation

of the antigen: a control experiment with the inactivated enzyme was also included. The usual course of 3 doses of 0.05 mg. of the antigen after treatment with trypsin was given to two rabbits, whereas two other animals received similar quantities of the control solution. The rabbits that received the antigenic material after it had been exposed to the action of trypsin both gave rise to a high titre (1 : 5000 and 1 : 2000 respectively) agglutinating serum. The two control animals likewise produced a serum giving agglutination at a high dilution. It would appear from these results that the immunological activity of the antigen is not destroyed by the action of trypsin at pH 8.5.

It seemed of interest to ascertain whether a commercial enzyme preparation, "luizyme", that is known to contain cellulases and hemicellulases, has any destructive action on the antigenic material. As far as could be determined there was no loss of antigenicity. Two rabbits that received the antigenic material after it had been exposed to the action of "luizyme" for 5 hr. at 37° produced immune sera giving agglutination at dilutions of 1 : 2000 and 1 : 1000 respectively, whereas the same antigenic preparation after it had been treated with the inactivated enzyme preparation induced the formation of immune sera each of which agglutinated the homologous organism at a dilution of 1 : 2000.

The toxicity of the antigenic substance

The minimum lethal dose of the most active fraction was determined by giving to a group of mice intravenous doses from 5.0 to 0.01 mg. of the substance dissolved in saline. The results of the animal tests are shown in Table VII. It

Table VII. *Showing the results of toxicity tests in mice with the antigenic material*

Antigenic preparation	Dose of preparation given intravenously mg.	Results of tests	
		No. inoculated	No. of deaths
(1) Purified by acetone and alcohol fractionation	0.50	3	3
	0.30	3	3
	0.20	8	8
	0.10	5	5
	0.05	5	1
	0.01	5	0
(2) Purified by extracting preparation (1) with tri- chloroacetic acid at 0°	0.50	3	3
	0.30	3	3
	0.20	8	8
	0.10	5	4
	0.05	5	0
	0.01	5	0

will be seen that 0.10 mg. represents 1 lethal dose of the preparation and that the material after treatment with trichloroacetic acid is similarly lethal in doses of this order. The mice which received these doses died within 12–18 hr. without showing any symptoms of paralysis, but severe diarrhoea is an early symptom. When 0.05 mg. of the antigenic preparation is given the mice are very ill for about 12 hr., but thereafter they completely recover without showing symptoms of paralysis. Rabbits inoculated intravenously with 5 mg. of the substance die within 24 hr. with diarrhoea, and a post-mortem examination may reveal haemorrhagic lesions in the wall of the caecum and intestines; paralytic symptoms appear to be absent.

A group of mice was given 2, 5 and 20 minimum lethal doses of a toxic autolysate of *B. dysenteriae* (Shiga). It was soon evident that even 20 lethal

doses failed to kill in less than 24 hr. whereas 5 doses required 48–72 hr. and 2 lethal doses, 72–120 hr. to bring about a fatal issue. The symptoms observed, particularly paralysis of the hind quarters, were those which are characteristic of a neurotoxin.

DISCUSSION

A method for the isolation of bacterial antigens which avoids the use of an acid or alkaline medium and which can be carried out at normal temperatures or at 0° has been developed in order to minimize the risk of changing the antigenic complex during the process of isolation and purification. Furthermore, the use of anhydrous organic solvents, dry bacteria and low temperatures also greatly reduces the possibility of enzymic action which in certain instances is known to cause considerable damage and even to destroy the antigenic complex completely. If the bacterial enzymes are of protein nature or are in some way closely associated with the protein, then the use of diethyleneglycol and certain of the other solvents will reduce the possibility of the enzymes being extracted along with the specific antigenic material.

If one may judge from the experimental work so far completed it would seem that very little somatic material other than the specific antigen is extracted from the bacillary body by the various solvents that have been employed. Indeed, only rarely has it been observed with *B. dysenteriae* (Shiga) that more than 1–2 % of the total dry weight of the organism passes into the solvent during each extraction and the percentage yield of soluble antigenic material even after several extractions does not exceed 6–7 % of the original weight of the mass of organisms. With other bacteria, for example, the “0” variant of *B. typhosum* the yield of the soluble antigenic material after fractionation with acetone may be as high as 9–10 % of the total dry weight of the organisms employed.

Whether it is possible to evolve a differential extraction method whereby several solvents are utilized and in this way to isolate the various antigens that are known to exist in some bacteria can only be decided by further work, but some preliminary experiments have already given encouraging results. In experiments of this nature the degree of solubility of the particular antigen in the selected solvent is not of primary importance for it may well be that a solvent which dissolves the antigen in question sparingly will prove to be a more suitable agent for its separation from other antigens associated with it than another solvent which readily dissolves the total antigenic material to a concentration of 1 or 2 %.

It is impossible to state with certainty that the preparations which have been obtained up to the present consist of a single chemical substance. The application of a variety of methods of fractionating the antigenic preparations has in each case ultimately yielded a substance with very similar chemical, physical and immunological properties and it seems probable that the material that has been obtained in this manner represents the essential bacterial antigen very largely freed from other non-specific substances. Qualitative tests indicate that the material is free from protein and, in support of this conclusion, it has been observed that trypsin fails to destroy the specific antigenic properties of the complex. The antigenic material is rapidly hydrolysed by 0.1N acetic acid at 100° and an examination of the hydrolysis products shows that about 48 % of the antigen consists of a specific polysaccharide; undoubtedly it is this constituent of the complex which endows the antigen, and hence the micro-organism, with its characteristic immunological specificity. The nature of the remaining portion of the antigen is as yet largely a matter of conjecture but it

appears to be in part lipoidal; it seems likely that a polypeptide chain may also be present since all preparations of the unhydrolysed material so far examined have given a definite biuret test but a negative ninhydrin reaction. Until further tests for homogeneity have been applied to the antigenic material it would be unprofitable to discuss in detail the probable chemical make up of the antigen molecule because, unless it is certain that the material designated as "antigen" is really a pure, homogeneous substance, the chemical properties assigned to it are of doubtful value. When discussing the immunological properties of the active preparations the position is somewhat different since it is improbable that contaminating non-specific substances—even if they are antigenic—can inhibit the production of specific immune body.

The antibacterial immunity response given by the antigen preparations appears to be qualitatively identical with that obtained when the whole micro-organism is employed. It has been apparent, however, that experimental animals tolerate the antigenic material much more readily than the highly toxic "Shiga" bacilli and in consequence they can be immunized more rapidly and finally yield immune sera of high titre. This is especially evident when the results of producing "Shiga" heterophile antibody are considered, since nearly every rabbit inoculated has produced an immune serum giving haemolysis up to a dilution of 1 : 500; titres of 1 : 1000–1 : 2000 have not been uncommon.

The results of the immunity experiments in which alkali-treated antigen was employed indicate that the antigenic structures associated with the production of specific agglutinins and precipitins are relatively resistant, whereas the corresponding structures that are bound up with the capacity of the antigen to induce the formation of "Shiga" heterophile immune body are alkali-labile. It has already been shown [Meyer & Morgan, 1935] that the action of alkali on the combining power of the polysaccharide portion of the antigen for the heterophile antibody is slight. These findings, therefore, when considered together suggest that the heterophile antigenic structures do not reside entirely in the polysaccharide portion of the antigen but are present also in an unidentified component of the antigenic complex. The reason for the close relationship that has been shown to exist between the power of the polysaccharide to combine with the antibacterial immune substance—agglutinin and precipitin—and its capacity to neutralize the heterophile antibody would appear to be that the polysaccharide combines solely with the antibacterial (antipolysaccharide) receptors on each type of antibody molecule and that in reality its molecule does not contain a specific heterophile antigenic structure. The answer to this interesting problem can only be obtained after further work has been carried out and experiments designed to contribute to its solution are now being undertaken in collaboration with Dr D. W. Henderson.

It is of interest to note that a horse which was immunized with the antigenic preparation produced 1.64 mg. of antibody protein per ml. of blood serum and therefore, if we assume a blood serum volume of 25 l., 41 g. of antibody protein has been formed during the immunization. The total antigen given to the animal was 16.2 mg. and the ratio of the amount of antigen injection to the quantity of antibody formed was approximately 1 : 2500. This figure must represent a minimum value because the antibody present in the tissues of the animal cannot be readily ascertained. The numerical value of the ratio, mg. of antigen injected/mg. of antibody produced, for rabbits that have been immunized was considerably smaller than the one obtained for the horse and was found to be, in the five sera examined, of the order 1 : 550, 1 : 550, 1 : 200, 1 : 400, 1 : 1000 respectively.

Olitsky & Kligler [1920] have produced evidence in support of the view that two independent toxic entities are associated with *B. dysenteriae* (Shiga). One of these is a soluble, heat-labile factor which is responsible for the production of paralysis in the experimental animal and is essentially a neurotoxin. The other type of toxin has been termed an endotoxin and is considered to be relatively heat-stable. Recently Boivin & Mesrobeanu [1937] and Mesrobeanu & Boivin [1937] have isolated two toxic constituents of *B. dysenteriae* (Shiga). One is a protein and corresponds to the soluble, heat-labile neurotoxin whereas the other toxic factor is heat-stable and is non-protein in nature. The results of our experiments dealing with the toxicity of the specific somatic antigen support the view put forward by these workers and indicate that this non-protein substance is identical with Olitsky & Kligler's endotoxin.

Antigenic fractions have also been isolated by the extraction method described from "Rough Vi", "O. Vi" and "O" strains of *B. typhosum*, and from other micro-organisms. A detailed study of the chemical and immunological properties of the various antigens contained in these organisms will be published in a later paper in collaboration with Dr D. W. Henderson.

SUMMARY

1. A method is described for the isolation of certain bacterial antigens which depends upon the solubility of the antigenic complex in a number of organic solvents. The process of extraction can be carried out at a neutral reaction and at normal or low temperatures.

2. Special reference is made to the isolation of the specific antigen which is associated with the "smooth" form of *B. dysenteriae* (Shiga).

3. This antigenic material is free from protein and is readily hydrolysed by dilute acid to yield about 48% of its weight of the specific polysaccharide, $[\alpha]_D + 98^\circ$; N, 1.8%. After acid hydrolysis a part (28%) of the antigenic complex is insoluble in water; this material contains 7.1% nitrogen and 0.8% phosphorus and is partly soluble in ether.

4. The antigenic material induces an antibacterial immunity response which is qualitatively identical with that produced by the specific antigen of the intact micro-organisms.

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