Studies in Immunochemistry

13. PREPARATION AND PROPERTIES OF THE 'O' SOMATIC ANTIGEN OF *SHIGELLA DYSENTERIAE* (SHIGA)

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The isolation and properties of an antigenic material which possesses the immunological character of the 'O' antigen of *Shigella dysenteriae* (Shiga) was described in a series of papers (Morgan, 1937, 1939, 1949; Morgan & Partridge, 1940, 1941; Partridge & Morgan, 1940). The results of this work indicated that the 'O' antigen consisted of a labile, poly-molecular complex which contained three main components; polysaccharide, conjugated protein and phospholipid. It was pointed out that although the results of the examination of the preparation indicated that the isolated antigenic material was largely freed from unspecific contaminants, the lack of knowledge of the behaviour of large molecular aggregates when subjected to the relatively crude and inefficient methods of purification employed did not enable a final decision as to the degree of homogeneity of the material to be reached.

The object of the present communication is to present the results of a re-examination of the problem of the isolation and characterization of the 'O' antigen of *Sh. dysenteriae* which includes a detailed study of the materials obtained by high-speed differential centrifugation.

MATERIALS AND METHODS

Organisms. *Sh. dysenteriae* (Shiga) (Type 1 of Kauffmann, 1951), strain K. 624 'Smooth' was used for the extraction of antigen, this strain had previously been used for this purpose by Morgan (1937) and Morgan & Partridge (1940, 1941). Cultures were carefully checked for purity before each large-scale growth, for suspension stability, and for smoothness of colonial morphology by plating on tryptic meat-digest agar. 'Rough' colonies were very rarely encountered in this strain.

Materials for analysis. For analysis, samples of extraction products were thoroughly dialysed against distilled water at 0–2° and dried from the frozen state. They were further dried to constant weight *in vacuo* at 78° before making into solutions of known (% w/v) concentration in water. The freeze-dried materials absorbed up to 15% of their weight of moisture when left exposed to the air.

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Total nitrogen. This was determined by the Kjeldahl method using the distillation apparatus of Markham (1942) and the mixed bromocresol green + methyl red indicator of Ma & Zuazaga (1942).

Phosphorus estimation. This was determined on samples containing 5–20 µg. of P by a modification of the Fiske & Subbarow (1925) method.

Sugars. Glucosamine was estimated by a modification of the method of Elson & Morgan (1933) on samples after hydrolysis with 0.5N acid. Rhamnose was determined by the method of Dische & Shettles (1948) on samples without previous hydrolysis.

Ultracentrifugation. Preparative ultracentrifuge runs were carried out in a Spinco Model 'L' Ultracentrifuge at 2–4°. Figures quoted for the force in *g* refer to the forces exerted at the centres of the tubes and represent average values. Analytical ultracentrifuge runs were carried out in a Spinco Model 'E' machine.

Antigenicity. Measured by the capacity of the materials to elicit the formation of specific *Sh. dysenteriae* (Shiga) agglutinins in the rabbit. Animals were injected intravenously with 0.5 ml. samples of fractions dissolved in sterile saline, generally three doses were given at 4-day intervals and the sera obtained a week after the last injection tested for the presence of agglutinins using a suspension of the heat-killed homologous organisms. The degree of agglutination was read after incubating the test serum and suspension for 2 hr. at 55° and leaving overnight at 0–2°. Normal rabbit sera rarely show agglutinins above a dilution of 1 in 2. This test was used to determine in which fraction the antigenic material was present through the purification process.

Toxicity. Toxicity was estimated by injecting groups of ten white mice (body wt. 18–22 g.) intraperitoneally for each of six dilutions of the test material in sterile saline. Deaths were recorded over 4 days, most of the animals which died did so on the second day.

EXPERIMENTAL AND RESULTS

Isolation of the somatic antigenic complex

Preparation of bacteria for extraction. The organisms were grown on solid 'CCY'-agar (Gladstone & Fildes, 1940) in enamelled steel trays, each containing 500 ml. of medium. After 22 hr. growth at 37°, the bacteria were reaped, filtered and washed quickly with saline and with water at 0–2°. The centrifuged cells were resuspended and killed by pouring into 10 vol. of cold acetone. After being checked for

sterility, the acetone was changed several times, the organisms were collected by centrifugation and dried *in vacuo*. Yield, about 400 mg. of dried organisms from each tray.

Extraction with diethylene glycol. The method used for the extraction of the antigenic material was essentially the same as that described by Morgan (1937) and by Morgan & Partridge (1940). In order to determine if the treatment of the organism with acetone before extraction with diethylene glycol, as used in the earlier work, had any deleterious influence on the yield or quality of the 'O' antigen, one preparation of living *Sh. dysenteriae* was dried from the frozen state and extracted directly with diethylene glycol. The yield and properties of the material soluble in the organic solvent did not appear to differ in any way from the product which was obtained when acetone-killed culture was employed. The technical difficulties and dangers associated with the production on a large scale of freeze-dried, living, virulent bacteria have not been satisfactorily overcome, and this type of culture preparation has, therefore, not been extensively used.

Acetone-dried bacterial cells (170 g.) were extracted with 10 parts (v/w) of freshly redistilled diethylene glycol. The suspension was shaken mechanically for several hours each day for 3–4 days at room temperature, methanol (10%, v/v) was added to reduce the viscosity and the cells were removed in the closed bowl of a Sharples centrifuge. The clear, yellow supernatant solution was passed through a Berkefeld filter candle, dialysed against distilled water at 2–4° until free from glycol, and the opalescent aqueous solution obtained was concentrated under reduced pressure at 16° until the solid content was about 1%.

The sedimented bacteria recovered from the Sharples bowl were resuspended in fresh diethylene glycol and re-extracted; four extracts were made. After the first extraction the cells were much more easily sedimented, and less glycol was used in the third and fourth extractions.

In order that the products of successive extractions could be compared, each was handled separately through the purification process.

Fractionation with acetone. Each of the four extracts was fractionated with acetone in the same way. The aqueous solution was cooled to 0–2° and acetone at –10° added slowly to a final concentration of 50% by vol. Some material was precipitated at this level and was removed by centrifugation. The acetone concentration was then increased to 66%, at which level the bulk of the antigenic material was precipitated. The precipitate was redissolved in water and treated with acetone a second time, a small amount of material was again removed at the 50% level and the antigen precipitated by increasing the concentration to 60%. The solution in water and precipitation with acetone was repeated a third time and the 50–60% acetone fraction finally obtained was redissolved in water, dialysed in the cold against distilled water and dried from the frozen state.

The yields, and the total N and total organic P contents of the four extracts are shown in Table 1. No inorganic P was present.

The material at this stage showed a definite absorption in the ultraviolet region at 260 mμ., which was due to the presence of nucleic acid and nucleoprotein. These materials could be removed by fractionation with (NH₄)₂SO₄.

Fractionation with ammonium sulphate. The products of the acetone fractionation of each of the four diethylene glycol extracts were dissolved separately in distilled water

to give 1% solutions, and each was fractionated with (NH₄)₂SO₄. Saturated (NH₄)₂SO₄ soln. at 20° was added to the antigen solutions at the same temperature and the precipitates formed were collected by centrifugation, washed with (NH₄)₂SO₄ soln. of the same percentage saturation as that which had precipitated the fraction and the washings were added to the appropriate supernatant solution. The fractionation scheme is set out in Fig. 1, from which it will be seen that each extract was divided into five parts separating

Table 1. *The 50–60% (v/v) acetone fractions of 'Shiga' diethylene glycol extracts*

No. of diethylene glycol extract	Yield (g.)	Total N (%)	Total P (%)	N/P
1	3.02	4.4	1.4	3.1
2	2.80	4.2	1.4	3.0
3	1.87	4.2	1.5	2.8
4	1.11	5.1	1.6	3.2

Fig. 1. Ammonium sulphate fractionation of the 50–60% (v/v) acetone fractions of 'Shiga' diethylene glycol extracts.

Table 2. *The ammonium sulphate fractionation of the 50–60 % (v/v) acetone fractions of diethylene glycol extracts of Shigella dysenteriae (Shiga)*

		Diethylene glycol extract			
		1st	2nd	3rd	4th
Original material	Wt. (g.)	3.0	2.8	1.8	1.0
	N (%)	4.4	4.2	4.2	5.1
	P (%)	1.4	1.4	1.5	1.6
Fractions					
P1 + P3 (0–35 %)*	Wt. (g.)	0.40	0.26	0.22	0.05
	N (%)	3.9	3.6	3.1	5.3
	P (%)	1.1	0.9	1.2	0.9
	N/P	3.5	4.0	2.7	5.6
P5 (35–38 %)*	Wt. (g.)	1.50	0.85	0.76	0.40
	N (%)	4.4	4.6	4.3	4.5
	P (%)	1.0	1.1	1.2	1.0
	N/P	4.4	4.2	3.6	4.5
S4 (38–40 %)*	Wt. (g.)	0.37	1.30	0.09	0.04
	N (%)	3.9	4.6	3.8	3.3
	P (%)	1.1	1.2	1.1	0.9
	N/P	3.5	4.0	3.5	3.6
S3 (40–42 %)*	Wt. (g.)	0.33	0.09	0.36	0.36
	N (%)	3.4	3.6	3.6	3.4
	P (%)	1.2	1.1	1.2	1.4
	N/P	2.8	3.3	2.9	2.4
S2 (soluble at 42 %)*	Wt. (g.)	0.11	0.09	0.08	0.03
	N (%)	5.9	6.2	6.6	6.3
	P (%)	1.4	2.2	2.6	2.5
	N/P	4.1	2.3	2.5	2.5

* % Saturation with ammonium sulphate.

at saturation levels of: below 35, 35–38, 38–40, 40–42 %, and that soluble at 42 %. Each fraction was dialysed at 0–2° until free from salt, and recovered by drying from the frozen state. The weights of the fractions obtained, together with their N and P contents, are given in Table 2. The materials precipitated at 35 % and those remaining in solution at 42 % saturation of salt were unstable in aqueous solution after recovery and both materials showed a strong ultraviolet absorption at 260 m μ , these fractions P1, P3 and S2 accounted for a third of the weight of the product of acetone fractionation. The other fractions gave stable opalescent aqueous solutions from which no material sedimented on prolonged centrifugation at 3000 rev./min. They showed no ultraviolet absorption which could be attributed to nucleic acid.

The four S4 fractions and the four P5 fractions had very similar N and P contents and were, therefore, pooled and reprecipitated with (NH₄)₂SO₄; no material came out of solution at the 35 %-saturation level and only a little remained in the 40 %-saturated supernatant fluid. The 35–40 % fraction was then precipitated twice with acetone, each time the 50–60 % (v/v) acetone fraction was kept and after dialysis and freeze-drying 4 g. was recovered, which contained 4.35 % N and 1.16 % P.

Extraction on a larger scale

Dry bacterial cells weighing 1 kg. were extracted four times with diethylene glycol by the method already described. In order that larger apparatus and vessels than one normally encounters in the laboratory would not be required, the batch was split into four parts, A, B, C and D, each weighing 250 g.

To avoid the difficulty of dialysing large volumes of diethylene glycol extracts and the subsequent concentration of the resulting dilute aqueous solutions, the extracts cooled to 0–2° were poured into several volumes of acetone at –15°, the acetone being recovered for use again. The precipitated material was redissolved in water to yield a 1 % (w/v) solution and fractionated with acetone as already described above. A yield of 99 g. of crude antigen was recovered between the concentrations of 50 and 60 % (v/v) acetone.

The acetone products were made up into batches of suitable size and fractionated with (NH₄)₂SO₄ as described above, except that the materials were collected in only three fractions, that precipitated at 35 % saturation, between 35 and 40 %, and that soluble in 40 %-saturated (NH₄)₂SO₄ solution. These materials were recovered by freeze-drying after dialysis in the cold against de-ionized water ('Permutit' de-ionizer). The main fraction precipitated in the range of 35–40 % saturation with (NH₄)₂SO₄ was again precipitated with acetone in the range of 50–60 % (v/v) and the product finally obtained weighed 53 g.

Examination of the materials obtained by fractionation with ammonium sulphate. Samples of the three fractions, dissolved in dilute alkali, were examined for absorption in the ultraviolet region. Both the side fractions showed strong absorption at 260 m μ . due to nucleoprotein (fraction below 35 %) and nucleic acid (fraction above 40 %), but the main fraction showed no maximum in this region. It had, however, a definite absorption at 245 m μ . and a weak one at 295 m μ . The absorption curves are shown in Fig. 2.

An examination of this material by a fractional-solubility test, as described later, showed that it was not homogeneous, and photographs obtained in the analytical ultracentrifuge

Fig. 2. The ultraviolet absorption spectra of the products of ammonium sulphate fractionation of 'Shiga' diethylene glycol extract (50-60%, v/v, acetone fraction). *A*, 35-40% fraction; *B*, material precipitated below 35%; and *C*, that remaining in solution at 40% saturation. *A* at 0.05%, *B* and *C* at 0.01% concentration. All materials in 0.01 N-NaOH.

showed that two components were present, one a rapidly sedimenting, polydisperse component and the other a slowly moving, sharp peak. Before attempting to separate these materials, however, the phospholipid component was removed.

Removal of the phospholipid

Morgan & Partridge (1940) showed that the antigenic complex contained a phospholipid component which could be readily dissociated and removed by the action of formamide without destroying the capacity of the material to induce the formation of specific agglutinins. A method used originally by Miles & Pirie (1939) for removing the material 'PLI' (a lipid + phospholipid mixture) from the *Brucella* antigen was, in the present experiments, employed in place of formamide to remove the phospholipid.

Material which had been fractionated with acetone and $(\text{NH}_4)_2\text{SO}_4$, and shown to be free from detectable amounts of nucleic acid, was dissolved in water to 1% concn., cooled to 0-2°, and poured into 10 times its volume of ether:ethanol (1:1, v/v) mixture containing 0.5% of conc. HCl, the solvent mixture being kept at -10°. The 'Shiga' antigen was precipitated and recovered by centrifugation at 0-2°, this was redissolved in water at 0°, the precipitation process repeated twice and the phospholipid-free material recovered. From 48 g. of antigen treated in this way, 41.5 g. was obtained which contained 4.35% N and 0.6% P.

Fractionation by differential ultracentrifugation

The phospholipid-free antigenic material (32 g.) was dissolved in 2.5 l. of water and fractionated by differential centrifugation. The solution was first centrifuged for 2 hr. at 40 000 *g* and the sediment (*P1*) was redissolved and freeze-dried. The supernatant fluid (*S1*) was centrifuged again for 2 hr. at 80 000 *g* the sediment (*P2*) was similarly recovered and the remaining solution (*S2*) centrifuged for a further 3 hr. at 105 000 *g*, giving a sediment *P3*. The supernatant solution remaining in the centrifuge tubes showed a sharp

boundary which separated a lower opalescent part, removed as fraction (*S3*), from an upper part (*S3a*) which was clear. The results of the analysis of these five fractions for N and P suggested a further series of centrifugations in which fraction *P2* was separated into a sediment *P2P* and supernatant *P2S* in 2 hr. centrifugation at 44 000 *g*, *S3* was separated into *S4* and *P4* at 105 000 *g*, and *S3a* into sediment *P5*, and fractions *S5a* to *S5c*, being the top, centre and lower parts, respectively, of the supernatant solution, after a further 3 hr. at 105 000 *g*. This fractionation scheme is set out in Fig. 3 with the weights and the N and P contents of the recovered fractions. Fractions *P3* and *P2S* were pooled to give a product (*SpM*) having N, 4.46; P, 0.75% and weighing 9.8 g. A further 4.4 g. of antigenic

Fig. 3. Fractional centrifugation of 'Shiga' antigen. N and P figures are given as wt. %. For explanation see the text.

Table 3. *Materials derived from antigen preparation SFa by ultracentrifugation*

Fraction	Wt. (g.)	Recovery (%)	N (%)	P (%)	N/P
Original material	4.40	—	4.1	0.63	6.5
Below 25000 g	0.36	8	6.35	0.90	7.0
25000 to 105000 g	2.47	56	4.6	0.88	5.2
Over 105000 g	1.53	35	2.3	0.19	12.1

Table 4. *Fractionation of 'heavy' material (fractions P1 and P2P) by differential centrifugation*

Fraction	Wt. (g.)	Wt. (%)	N (%)	P (%)	N/P
P1/1	0.59	8	6.9	0.88	7.85
P1/2	0.39	5	6.4	0.85	7.5
P1/3	2.27	31	6.2	0.85	7.3
P1/4	4.14	56	5.2	0.75	6.7

material (*SFa*), after removal of phospholipid, was fractionated in the centrifuge in a simpler way and gave the products detailed in Table 3.

It is evident that, after fractionation with acetone and $(\text{NH}_4)_2\text{SO}_4$ and removal of the ether-soluble phospholipid, the antigenic material contains three components. The first of these is not sedimented at 100 000 g under the conditions studied and contains about 2.2% N. This fraction has been purified and obtained in an homogeneous condition as shown by the methods of fractional solubility, electrophoresis and ultracentrifugation, and will be described in greater detail in a later communication; it is the specific, undegraded polysaccharide, free from the other components which make up the native antigenic complex. The material is not antigenic in the rabbit and not significantly toxic, and differs in some important respects from the undegraded polysaccharide which can be obtained from the antigenic complex by dissociation (Morgan & Partridge, 1941). The second material contains 4.5–4.6% N, sediments between 40 000 and 105 000 g, gives a stable, opalescent aqueous solution, and is a powerful antigen. The third material contains about 6% N, is thrown out of solution at 40 000 g and fails to give a stable solution when resuspended in water.

Two of the most readily sedimented 'heavy' fractions (*P1* and *P2P*, Fig. 3) were pooled, giving 7.45 g. of material containing N, 5.7; P, 0.79%. This was fractionated by differential centrifugation and gave four fractions, *P1/1* to *P1/4*. *P1/1* sedimented at 5000 g, *P1/2* at 10 000 g, while *P1/3* and *P1/4* were the heavily opalescent bottom half, and the almost-clear top half of the supernatant solution, respectively. The weights and the N and P contents of these fractions are shown in Table 4. Fraction *P1/2* (300 mg.) was suspended in water and, after being allowed long enough for as complete solution as possible, was centrifuged at 5000 g. The opalescent supernatant solution was removed, the sediment resuspended in water and centrifuged again. This process was repeated three times in all. The final supernatant was clear and did not appear to have any material contained in it. However, the pooled supernatant solutions subsequently yielded 72 mg. of material which contained N, 4.25; P, 0.9%, and which was closely similar to the soluble antigen. The final water-insoluble fraction weighed 193 mg. and contained N, 7.1; P, 0.8%. A sample of this

material (93 mg.) was suspended in 1% (v/v) acetic acid and heated for 3 hr. at 100°, after this time the material remaining in suspension was removed by centrifugation, leaving a clear solution from which the degraded specific polysaccharide was precipitated with 10 times its vol. of ethanol; 21 mg. was recovered, or about 23% of the weight of the water-insoluble material used. The amount of degraded polysaccharide recoverable from the soluble antigen (N, 4.5%) after hydrolysis under similar conditions is 65–70% (see below).

Examination of the main antigenic fraction (SpM)

A sample (400 mg.) of fraction *SpM* (Fig. 3) was examined for homogeneity in a fractional solubility test as follows. The material was suspended in 100 ml. of redistilled propylene glycol, in which it is sparingly soluble, and the suspension shaken mechanically. After 24 hr., when part had dissolved, the suspension was centrifuged and the supernatant solution removed. A fresh volume of solvent was added to the sediment and the process repeated until nearly all the material had dissolved. After 12 days, the material had been divided into 12 fractions, which were pooled in successive pairs and the materials recovered by pouring each dissolved fraction into several times its volume of acetone, washing until free from glycol and drying from the frozen state. Analytical figures for these fractions are shown in Table 5.

No significant differences can be seen between the analyses of the different fractions, whereas before fractional centrifugation, although no differences were found in the different fractions obtained in a solubility test using water as solvent, a significant rise was found in the N content of successive fractions in propylene glycol. This test, therefore, does not reveal any evidence of lack of homogeneity in the material investigated.

The material, *SpM*, was examined electrophoretically at pH's 4.1 and 8.0. The substance travelled very slowly and showed a small amount of a second component. Examination of the material in the analytical ultracentrifuge also showed that two components were present both at pH 4 and at pH 8. The major component travelled fast and was somewhat polydisperse. The second material, present in small amount only, gave a slowly moving, sharp peak, later identified as free, undegraded polysaccharide.

Table 5. *A fractional solubility test on antigenic material (fraction SpM) using propylene glycol*

Fraction	Wt. (mg.)	N (%)	P (%)	N/P	Rhamnose (%)	Glucosamine (base) (%)
1	109	4.4	0.75	5.9	22.4	14.6
2	215	4.4	0.75	5.9	22.2	14.6
3	125	4.3	0.74	5.9	22.9	14.6
4	120	4.55	0.75	6.05	21.8	13.6
5	115	4.4	0.75	5.9	21.7	14.2
6	248	4.55	0.78	5.85	21.7	14.0

Table 6. *Materials obtained by fractional centrifugation of pooled antigen preparations*

Fraction	Sedimentation range (g)	Wt. (g.)	N (%)	P (%)
Sp2/3	25 000–80 000 (sedimented in 6 hr.)	1.25	4.53	0.83
Sp3/1	80 000–105 000 (sedimented in 3 hr.)	1.05	4.56	0.82
Sp3/2/1	105 000 (supernatant solution from Sp3/1 sedimented in 6 hr.)	1.50	4.50	0.80

Further fractional centrifugation

Fractions *SpM* (Fig. 3), *SFa* (middle fraction, Table 3) and *P1/4* (Table 4), totalling 12 g., were pooled, dissolved in water, and centrifuged in such a way as to give three main antigen fractions sedimenting in different ranges of *g*, and such that each of the fractions was centrifuged at least twice at the lower force to remove the heavier material and at least 3 times at the higher force to remove lighter materials. Analytical details of these three fractions are given in Table 6. The amounts of material obtained in these fractions do not represent the amount present in the starting material, the remainder, present in the side fractions, was not recovered in this series of runs owing to the amount of centrifuging this would have entailed. The analysis of these fractions shows that although the antigenic complex is polydisperse, materials sedimenting at different levels of *g* and differing, therefore, in particle size, have most probably the same chemical composition.

Examination of materials for homogeneity

Fraction *Sp 3/1* (Table 6) was examined for homogeneity in the analytical ultracentrifuge (Fig. 4). At 0.5% concentration and pH 4.5, the material showed only one component, but at 1.25% concentration a slower-moving, second component was still visible (Fig. 4*b*), though present in very small amount. At pH 8.0 the amount of this material, which was identified as free polysaccharide, had increased to several per cent (Fig. 4*c*). At pH 11 the free polysaccharide had increased still further in amount (Fig. 4*d*) and the separation of a further material from the main component is revealed. The amount of free polysaccharide present, at least in

the physiological range of pH, is so low that it cannot be detected by other means. Further centrifugation of a sample of fraction *Sp 3/1* gave a material (*Sp 3/1/1*) which showed no detectable change in N and P content but in which no slowly moving second component (free polysaccharide) could be demonstrated at pH 7 (Fig. 4*a*). In view of

Fig. 4. Sedimentation patterns of 'Shiga' antigen fractions. Force, 30 000 g. Migration from right to left. (a) *Sp 3/1/1* at pH 7 (phosphate:NaCl, *I*=0.2), (b) *Sp 3/1* at pH 4.5 (acetate:NaCl, *I*=0.2), (c) *Sp 3/1* at pH 8 (phosphate:NaCl, *I*=0.2), (d) *Sp 3/1* at pH 11 (glycine:NaCl). Concentrations: (a) 1.2% (w/v), (b), (c), and (d) 1.25% (w/v). Exposures (a), (b) and (c) at 59 min., (d) at 91 min.

the amount of centrifuging required to obtain a larger amount of similar material, fractions *Sp* 3/1, *Sp* 2/3 and *Sp* 3/2/1 (Table 6) were used without further treatment in most of the experiments described below.

Properties of the antigenic complex

General. The materials dissolved in water to give opalescent solutions, the degree of opalescence depending on the range of *g* in which the fractions were sedimented. Measured as the percentage light absorption of a 1 cm. depth of a 0.5% solution in water, fractions *Sp* 3/2/1, *Sp* 3/1 and *Sp* 2/3 (Table 6) gave figures of 51, 61 and 68, respectively. Fraction *Sp* 3/1 had $[\alpha]_D$ $66 \pm 2^\circ$ in water, and the viscosity measured in acetate buffer, pH 4.5 ($I = 0.1$, containing 0.1M-NaCl), was 1.35 in 1.25% solution and 1.08 in 0.5% solution, relative to the buffer at 20°. Dissolved in borate buffers of pH's 8.5–9.5, the antigen showed marked dilatancy, the mobile solution becoming a firm gel on slight shaking, reverting to a mobile solution on standing for a few minutes.

Although the antigen after drying from the frozen state failed to dissolve in diethylene glycol it was, nevertheless, readily soluble in ethylene and trimethylene glycols. A stable, clear solution of the antigenic complex in diethylene glycol could be obtained by first dissolving the material in water and subsequently replacing the water by dialysis against diethylene glycol.

Electrophoresis. Examination of a purified antigen preparation, *Sp* 3/1/1, in the electrophoresis apparatus showed that migration was very slow, and somewhat slower at pH 4.1 than at pH 8.0; diffusion was also slow, however, and this allowed appreciable movement of the boundaries to be observed up to 48 hr. Opalescence of the antigen solutions prevented examination at higher concentrations. Exact mobility measurements were not made. Photographs obtained after 24 and 30 hr. at pH's 8.0 and 4.1 are shown in Figs. 5*a* and 5*b*, respectively. Only one component is demonstrable at both pH values.

Stability. The antigenic material essentially free from unbound specific undegraded polysaccharide showed no increase in the amount of the latter component on re-examination in the analytical ultracentrifuge after several months' storage in the freeze-dried condition at room temperature. A 1.25% solution of the material in phosphate buffer at pH 7, after storing for 30 days at 0–2°, showed no change when re-examined in the ultracentrifuge.

Particle size. The results of examination of the material in the ultracentrifuge indicate that dissociation of the complex takes place in alkaline solution and that as the pH rises the main component travels more rapidly.

Sedimentation constants were determined using

fraction *Sp* 3/1 in acetate buffer pH 4.5, $I = 0.1$ containing 0.1M-NaCl and phosphate buffer, pH 8.0 ($I = 0.1$, containing 0.1M-NaCl). The results obtained are shown in Table 7. The partial specific volume measured pycnometrically in acetate buffer, pH 4.5 ($I = 0.1$, containing 0.1M-NaCl) was determined at 2% concentration and found to be 0.712. The S_{20} values plotted against concentration give curves which rise steeply towards the S_{20} axis and cannot be extrapolated, neither could sedimentation constants be obtained with solutions below 0.3% concentration owing to rapid spreading of the boundary owing to the material being somewhat polydisperse. The curves obtained by plotting $1/S_{20}$ against concentration could be extrapolated, and the pH's 4.5 and 8 data converged to approximately 0.007 at infinite dilution. Considering the experimental errors involved and that only three points were available to draw each curve, it is thought that the sedimentation constant extrapolated to infinite dilution is probably independent of pH and lies in the region of 130–150. The mean particle weight of the material would, therefore, appear to be of the order of 1×10^7 .

Fig. 5. Electrophoresis diagrams of 'Shiga' antigen fraction *Sp* 3/1/1, (a) at 0.30% concentration, pH 8 (phosphate, $I = 0.2$), (b) at 0.35% concentration, pH 4.1 (acetate, $I = 0.1$). Exposures at 24 hr. and 30 hr., respectively. Direction of migration shown by arrows.

Table 7. Sedimentation constants of 'Shiga' antigen preparation (fraction *Sp* 3/1)

pH	Concn. (%)	$S_{20} \times 10^{-13}$ (corr.)	$1/S_{20}$
4.5	1.25	40	0.025
4.5	0.5	84	0.012
4.5	0.3	104	0.010
8.0	1.25	29	0.034
8.0	0.5	67	0.015
8.0	0.3	91	0.011

Chemical properties. Analytical figures obtained using antigen preparation *Sp* 3/1 are: C, 44.3; H, 7.04; N, 4.56; P, 0.82; ash, 3.1; rhamnose, 22 ± 1 ; glucosamine (base), 14.3 ± 1 %.

Mild hydrolysis of the material in 1 % (v/v) acetic acid at 100° for 3–4 hr. in an atmosphere of N₂ gas causes the antigenic complex to separate into protein and degraded-polysaccharide components. The chemical properties of these degradation products will be given in a subsequent communication. The acid-insoluble protein is recovered by centrifugation, washing and drying from the frozen state and amounts to 20–25 % of the weight of the antigen. The degraded polysaccharide is recovered from the acetic acid supernatant fluid by precipitation with ethanol and amounts to 60–70 % of the antigen used.

Biological properties. Specific precipitation could be obtained using a sample of purified antigen (*SpM*) at a dilution of 1 in 200 000. This test was carried out in a volume of 0.25 ml. using a rabbit serum prepared by the injection of six doses of purified antigen each of 10 µg.; the tubes were incubated at 37° for 2.5 hr. and the degree of precipitation observed after the tests had stood overnight at 0–2°.

The agglutination titres obtained with some of the products are shown in Table 8, together with the N and P contents of these fractions, the titres are average figures of those obtained with sera from four animals. Mixed fractions containing antigen and free specific undegraded polysaccharide, i.e. those with a low N and P content, are seen to be less antigenic than the purified materials (N, 4.4–4.6 %) and when a purified sample of the polysaccharide was injected into two rabbits in three doses amounting to a total of 100 µg., no agglutinins

could be detected in the resulting sera when tested at a dilution of 1:2.

The water-insoluble fractions with a high N content were dissolved in dilute sodium carbonate and diluted with saline for injection, these materials proved to be strongly antigenic.

The toxicity of some fractions is given in Table 9; the highly purified material, *SpM*, had a LD₅₀ (median lethal dose) value of about 80 µg. in the mouse. The lethal dose is seen to increase with decreasing N and P content of the materials and fraction *S5c* which is very largely undegraded polysaccharide is of relatively low toxicity.

Extraction experiments with additional strains of Shigella dysenteriae (Shiga)

It has been found, in agreement with the finding of Morgan & Partridge (1940), that extraction of a 'Rough' strain of *Sh. dysenteriae* (Shiga) with diethylene glycol yields a small amount of material only, which, if contained in the extract obtained from a similar quantity of 'Smooth' culture would contaminate the somatic antigen preparation to a negligible extent. Thus, from 66 g. (dry wt.) of 'Rough' organisms (K. 624 R) 960 mg. of material was extracted with the diethylene glycol, but only 20 mg. of material (N, 6.3; P, 1.95 %) was recovered in the 50–60 % acetone fraction.

A highly agglutinable strain of *Sh. dysenteriae*, recently isolated from a patient, was grown in bulk, and 145 g. (dry wt.) yielded 0.4 % of its weight in the 50–60 % acetone fraction, this contained N, 5.8; P, 1.9 %. It was reported earlier (Schütze, 1944) that highly agglutinable, 'Smooth' strains of this species gave a low yield of antigen, while inagglutinable strains gave a high yield. Strain K. 624 'S' is a relatively inagglutinable strain.

Extraction with trichloroacetic acid. A batch of acetone-dried organisms weighing 25 g. was extracted with 0.25N trichloroacetic acid for 3 hr. in the cold according to the method of Boivin, Mesrobian & Mesrobian (1933). The primary extraction product was precipitated with 6 times its volume of cold ethanol three times and the final precipitate redissolved, dialysed and freeze-dried. The product weighed 700 mg. (a yield of 2.8 %) and it contained N, 2.6; P, 0.5 %. This material gave an antiserum in the rabbit on injection of 3 µg., which agglutinated the homologous organisms to a titre of 48 (average of four animals). This titre is about that expected of a material containing 2.6 % N, if one assumes it to be a mixture of true antigen and undegraded polysaccharide (see Table 8). Examination of this material in the analytical ultracentrifuge showed this to be the case; a small, polydisperse peak could be seen due to the antigen and a large, slowly moving, sharp peak due to the polysaccharide.

Table 8. Agglutination titres of sera obtained by intravenous injection of 'Shiga' antigen fractions into rabbits

Fraction	N (%)	P (%)	Total dose (µg.)	Titre (average from four rabbits)
<i>P2P</i>	6.0	0.83	3	128
<i>SpM</i>	4.46	0.75	3	320
<i>S4</i>	2.7	0.50	3	48
<i>SFa</i>	4.6	0.88	1	20
<i>P1/1</i>	6.9	0.88	30	2560
<i>S5c</i>	2.3	0.10	30	10

Table 9. Toxicity of antigen fractions in mice

Fraction	N (%)	P (%)	LD ₅₀ (µg.) and 95 % confidence limits
<i>SFM</i>	4.35	0.60	52... 80...123
<i>SFd</i>	4.05	0.51	56... 86...131
<i>S3</i>	3.0	0.45	100...160...270
<i>S5c</i>	2.3	0.10	220...370...620

A sample of this product (400 mg.) was sedimented 3 times in the preparative ultracentrifuge at 105 000 *g*, each time the supernatant was carefully removed and the precipitate resuspended. The final precipitate weighed 48 mg. (12% of the whole) and contained N, 4.5; P, 1.0%. These figures are close to those found for the purified product extracted with diethylene glycol.

DISCUSSION

From a culture of the 'Smooth' form of *Sh. dysenteriae* (Shiga), a material has been obtained by extraction with diethylene glycol, acetone and ammonium sulphate fractionation, removal of phospholipid and differential high-speed centrifugation; this material induces the formation of agglutinins in the rabbit on injection of 1 μ g. and kills 50% of mice at a dose of 80 μ g. The antigenic material consists of a serologically specific polysaccharide associated with a protein moiety.

The removal of the last traces of free, undegraded specific polysaccharide from the antigenic material was accomplished with difficulty and the amount of centrifugation required to achieve this end did not allow the large preparations to be completely freed from the polysaccharide. The amount remaining, however, is such as to be detectable only when relatively concentrated solutions (over 1%, w/v) are examined in the analytical ultracentrifuge; its presence has not been revealed by other means. This undegraded polysaccharide, when freed from the antigenic complex, is neither antigenic in rabbits nor toxic in mice.

The phospholipid-free antigen preparations before fractionation in the preparative ultracentrifuge contained, in addition to the free undegraded polysaccharide, a material which possessed a relatively high nitrogen content (6-7%), and would not redissolve in water after separation in the centrifuge at fairly low speed (25 000 *g*). The fact that this was stable in aqueous solution in the presence of the other components of the mixture before separation suggests some kind of weak, physical interaction between the different molecular species present. This 'heavy' material was a powerful antigen and appeared to be qualitatively the same as the soluble antigen, but contained relatively more protein and less of the specific polysaccharide component, and was in a state of greater aggregation.

The analytical figures of the fractions obtained in the first main centrifuge separation (Fig. 3) suggested the possibility that the antigenic material was composed of a range of molecules of varying sizes and composition, from large ones having a relatively high nitrogen content to small ones composed largely of specific polysaccharide. The final results show, however, that this conclusion is

probably not correct. The antigenic complex sedimenting within the range 25 000 *g*-105 000 *g* shows remarkably constant analytical figures (4.5-4.6% N and 0.80-0.85% P) and the same figures were found for three fractions, *Sp* 3/1, *Sp* 2/3 and *Sp* 3/2/1, which were sedimented with different forces.

Examination in the analytical ultracentrifuge of one of the most carefully prepared antigen fractions (*Sp* 3/1) indicated that at pH 4.5 the material was in a state of greater aggregation than at higher pH values; on extrapolation to infinite dilution, however, the sedimentation constant did not differ significantly from the figure found using the data obtained from the examination at pH 8.

Dissociation of the antigenic complex in alkaline solution is evident in the ultracentrifuge patterns (Fig. 4*b-d*). At no time during the extraction and purification, however, is the material exposed to alkaline conditions. The re-examination in the ultracentrifuge of the purified product after storage in the freeze-dried condition for several months and after storage as a 1.2% (w/v) solution in phosphate buffer at pH 7.0 for 30 days failed to demonstrate that any physical changes had occurred.

The material travels slowly during electrophoresis and shows little diffusion after 48 hr., although the conjugated protein component, which will be described later, is itself highly charged and migrates rapidly.

An examination of the product obtained by extracting organisms with trichloroacetic acid according to the procedure described by Boivin *et al.* (1933) reveals that this preparation is composed of undegraded polysaccharide and antigenic material which has a nitrogen and phosphorus content similar to that found in the diethylene glycol product. The amount of antigenic material in the trichloroacetic acid extract is, however, relatively small.

SUMMARY

1. Acetone-dried cells of *Shigella dysenteriae* (Shiga) were extracted with diethylene glycol and a preparation of the somatic 'O' antigen was recovered. The material was purified by fractionation with acetone and with ammonium sulphate.

2. The phospholipid component was removed from the antigenic material and the resulting polysaccharide-protein complex fractionated by high-speed centrifugation.

3. A heavy, water-insoluble material was recovered at 25 000 *g*; this was strongly antigenic.

4. Uncombined, undegraded, specific polysaccharide was present in the original diethylene glycol extracts of *Sh. dysenteriae*, and was not deposited after prolonged centrifugation at 105 000 *g*.

5. The antigen fractions separating between 25 000 and 105 000 *g*, contained 4.5-4.6% N and

0.80–0.85 % P, were free from detectable impurities as shown by fractional solubility tests, electrophoretic examination and behaviour in the analytical ultracentrifuge at pH 7.0.

6. Specific polysaccharide dissociates progressively from the antigen at alkaline pH values.

7. Sedimentation constants at pH's 4.5 and 8 obtained for the phospholipid-free antigen and extrapolated to infinite dilution give approximately the same figure (0.007), which indicates a particle weight of the order of 1×10^7 .

8. The material possesses an LD₅₀ of 80 µg. in the mouse and induces formation of 'Shiga' agglutinins in rabbits on the injection intravenously of 1 µg.

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Comparative Studies of 'Bile Salts'

7. BILE ACIDS OF THE COYPU, *MYOCASTOR COYPU*

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Myocastor coypus is a vegetarian rodent of South American origin which, bred for its fur (nutria), has become acclimatized in the wild state in this country and elsewhere in Europe (Laurie, 1946). Brigl & Benedict (1933) reported that the bile of this animal contained a unique bile acid, 'nutriacholic acid', conjugated with glycine; this claim was supported by Haslewood & Wootton (1950). Prof. Shimizu of Okayama University, in a letter to the present author dated February 1952, stated that Kazuno in 1946 had shown that 'nutria glycocholic acid' was a conjugate of 3-hydroxy-7-oxocholanic acid and glycine, and that coypu bile also contained ursodeoxycholic acid (3 α :7 β -dihydroxycholanic acid). At that time work on coypu bile in these laboratories had been continued and it was decided to attempt a comprehensive study of the bile acids. At the same time, the opportunity was to be taken of testing new methods for the purification and identification of such substances.

RESULTS

It was at once found that Kazuno's claim was well founded; 'nutriacholic acid' was identical with 3 α -hydroxy-7-oxocholanic acid (III), prepared in good yield by partial oxidation of chenodeoxycholic acid (I). It was also found that condensation of this ketonic substance, as its ethyl ester, with Girard's reagent 'T' was not quantitative; nine separate Girard processes were required to extract it almost completely from the mixed ethyl esters obtained from the bile salts. In the non-ketonic material left after these experiments, esters of the following were identified: cholic (3 α :7 α :12 α -trihydroxycholanic) acid, chenodeoxycholic (3 α :7 α -dihydroxycholanic) acid (I) and ursodeoxycholic (3 α :7 β -dihydroxycholanic) acid (II); the isolation of derivatives of these substances was greatly facilitated by the paper chromatography described below, and the ethyl esters of the last two compounds were actually first