128. STUDIES IN IMMUNOCHEMISTRY

6. THE USE OF PHENOL AND OF ALKALI IN THE DEGRADATION OF ANTIGENIC MATERIAL ISOLATED FROM *BACT. DYSENTERIAE* (SHIGA)[1]

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EARLIER work has shown [Morgan, 1937; Morgan & Partridge, 1939, 1, 2; 1940, 1; Partridge & Morgan, 1940] that the antigenic substance which is responsible for the dominant serological properties of the 'smooth' form of *Bact. dysenteriae* (Shiga) can be extracted from the micro-organism by means of diethyleneglycol. The properties of the antigenic complex were described in some detail and three major components were isolated and identified as polysaccharide, phospholipin and a protein or polypeptide-like substance. By the action of cold, neutral formamide, the phospholipin component was removed and there remained a complex of specific polysaccharide and the polypeptide-like component which still possessed powerful antigenic properties. The material dissolved in phosphate buffer (*p*H 7·5–8·0) solution containing 0·85% NaCl to yield a clear 0·2% solution which could readily be passed through a sterile Berkefeld candle without appreciable loss. The protein component of the original antigenic complex on the other hand could be largely removed by the action of trypsin but the resulting polysaccharide-phospholipin complex possessed no significant antigenic capacity, as measured by the production of 'Shiga' agglutinins and precipitins.

A number of other experimental procedures designed to separate the components of the antigenic complex were also mentioned. The material was shown to yield clear solutions in cold anhydrous formic acid and in concentrated solutions of phenol. From these solvents the antigenic material could be largely recovered by precipitation with alcohol or acetone. Details of our work with the latter solvent are now given with some observations on the action of alkali on the antigenic complex.

EXPERIMENTAL

It has already been shown [Morgan & Partridge, 1940] that the protein or polypeptide-like component of the antigenic complex of *Bact. dysenteriae* (Shiga) can be removed by the action of trypsin or by the dissociating action of anhydrous formamide. The experimental work described below extends these observations and indicates that this component can also be eliminated from the antigenic complex by the action of phenol or by repeated precipitation of the complex from alkaline solution by alcohol. All these procedures, however, destroy the capacity of the resulting substance to induce the formation of 'Shiga' agglutinins and precipitins. As a result of the work to be described in this paper the so-called polypeptide-like component of the antigenic complex is now considered to be a conjugated protein and therefore the former term 'polypeptide-like' component will no longer be used.

[1] For Part 5 see Partridge & Morgan [1940].

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The action of phenol on the antigenic complex

In some early experiments attempts were made to remove the protein component of the antigenic complex by thoroughly extracting a 2–3 % aqueous solution of the primary diethyleneglycol extraction product [Morgan & Partridge, 1940, 1] with a strong solution of phenol. The upper aqueous layer was removed and again treated with liquid phenol but the product finally recovered from the aqueous layer still contained protein and represented a large proportion of the original material. Examination of the resulting product revealed, however, that the phospholipin component had been removed during the phenol extraction.

During some attempts to separate from crude preparations of the antigen, agar or agar hydrolysis products, such as kanten, by means of their insolubility in 90 % liquid phenol, it was observed that the antigenic complex was readily soluble in phenol of this strength and could be recovered from phenol solution by precipitation with alcohol. At first no attempt was made to fractionate the phenol-soluble material but it was noticed that after several treatments with phenol in this manner there was a significant decrease in the nitrogen content of the product finally isolated. Examination of the successive alcoholic phenol mother liquors showed that the amino-acid complex was being gradually eliminated. It was also observed that the antigenicity of the complex recovered after phenol treatment was considerably reduced, presumably owing to the elimination of the protein, and for this reason the degradation process was not extended, at that time, to eliminate the whole of the protein component. At this stage in the investigation Palmer & Gerlough [1940] described a method whereby the 'O' antigenic complex of *Bact. typhosum* could be recovered from the dry bacterial cells or from antigenic preparations by extraction of the material with 88 or 95 % liquid phenol. The antigenic complex remained insoluble in phenol of this strength. Our earlier experiments on the action of phenol on the antigenic complex of *Bact. dysenteriae* (Shiga) were continued, using a technique very similar to the one described by these authors.

Dissociation of the antigenic complex. A preparation (1·25 g.) of the antigenic complex of *Bact. dysenteriae* (Shiga), $[\alpha]_{5461} + 65 \pm 5°$, N, 4·4 %, after treatment with formamide to remove the phospholipin component, was treated with two quantities of 30 ml. of 90 % phenol. The material dissolved completely and could be recovered from the combined phenol solutions by the addition of alcohol. The greater part (0·95 g.) of the dissolved substance was thrown out of solution between the alcohol levels 0 and 33 % by volume. There was no precipitate formation on increasing the alcohol concentration from 33 to 50 %. The alcoholic supernatant was dialysed to remove phenol and alcohol and the resulting aqueous solution was taken to dryness *in vacuo*. This fraction gave strong biuret and Sakaguchi tests. The material thrown out of 90 % phenol solution between 0 and 33 % alcohol levels contained 3·4 % N and 3·8 % ash. It was again treated with 90 % phenol but the volume of phenol solution was reduced so that a small amount (5–10 %) of the material was left undissolved. Once again the main portion of the substance was recovered from the phenol solution by the addition of alcohol to 33 %. The material showed $[\alpha]_{5461} + 68 \pm 4°$, N, 3·0 %, P, 0·64 %. The fraction was then extracted with 92 % phenol (30 ml.) in an attempt to separate a larger phenol-insoluble portion. The insoluble material weighed 0·32 g. and contained 3·1 % N, 0·64 % total P and no inorganic P. The composition is seen to be very similar to that of the 90 % phenol-soluble material which was precipitated by the addition of alcohol up to 33 %. A 0·5 % aqueous solution of the material insoluble in 92 % phenol was too opalescent for accurate deter-

mination of specific rotation. The material precipitated from solution in 92% phenol by 33% alcohol gave the following analytical data: $[\alpha]_{5461}+72\pm3°$; N, 2·97%; P, 0·77%; inorganic P, nil. A quantitative Sakaguchi test carried out according to the method of Jorpes & Thorén [1932] showed the material to contain at least 0·15% arginine. The phenol extraction of the material insoluble in 92% phenol was repeated twice but each successive phenol-insoluble portion showed a similar rotation and N content and the Sakaguchi colour reaction indicated that in each preparation about 0·15–0·30% of arginine still remained in the phenol-insoluble fractions. The analytical figures for the material showed $[\alpha]_{5461}+76\pm4°$ (*c*, 0·5) N, 2·95%; P, 0·75%.

After the above phenol extraction experiments had been completed work on the recombination of the non-antigenic specific polysaccharide and protein components of the 'Shiga' antigenic complex [Partridge & Morgan, 1940] indicated that from solution in certain organic solvents these components could be precipitated in the form of an antigenic complex that possessed chemical, physical and immunological properties very similar to those of the original polysaccharide-protein complex derived from the native antigen. These observations suggested that the recovery of phenol-soluble and dissociated material by precipitation with alcohol caused the free polysaccharide and protein components to recombine for the most part, and thus the material precipitated from phenol solution by alcohol was composed to a considerable extent of a complex of these components and not of a simple mixture of polysaccharide and protein. The alcoholic phenol supernatant fluid, however, usually contained some of the free protein component of the antigenic complex which could be readily recovered after dialysis of the fluid to remove alcohol and phenol. The existence of the uncombined amino-acid complex in the alcoholic phenol offers an explanation for the lower protein content of the material precipitated from phenol solution by alcohol.

The experiments described above failed to show, in a clear cut manner, whether the protein component could be preferentially extracted from the antigenic complex by simple leaching with phenol solution. In order to obtain further information on this point, the experiment was repeated using a 95% phenol solution for extraction. The antigenic material was only sparingly soluble in this solvent and thus, even after several extractions with phenol, a considerable part of the complex remained undissolved and was therefore available for analysis and for antigenicity tests.

A preparation of the primary diethyleneglycol extraction product was thoroughly treated with cold anhydrous formamide (M.P. 2°) to remove the phospholipin component and, as a result of the thorough treatment, the product was found to have lost part of its polysaccharide as well as the phospholipin component. The analytical figures were 6·4% N; 1·4% P; inorganic P, nil; ash, 5·0%. A similar polysaccharide-protein complex containing a high proportion of the latter component has already been described in detail [Morgan & Partridge, 1940, 1]. The specific rotation of the material could not be determined with accuracy owing to a heavy bluish opalescence but was estimated to be $+30\pm5°$. It has frequently been observed that preparations of antigenic material possessing a high content of the protein component show an intense bluish opalescence when in aqueous alkaline solution.

The phospholipin-free material (1·0 g.) was treated with 30 ml. of 95% phenol solution and after thorough shaking was set aside for 30 min. at room temperature. The material swelled up to a thin transparent jelly but was only sparingly soluble in the solvent. The suspension was centrifuged and the

insoluble material was resuspended in fresh 95% phenol. After an hour the bulk of the preparation was again recovered and resuspended in fresh phenol solution. The process of extraction with phenol was repeated seven times. Methyl alcohol was added to each phenol supernatant fluid to make a concentration of 3% and each mixture was dialysed separately until free from phenol. The addition of alcohol produced no precipitate and was made to prevent the strong phenol solution from crystallizing during manipulation and to increase the rate of diffusion of phenol through the cellophane. Some material insoluble in dilute phenol solution separated during the later stages of dialysis and finally remained insoluble after the phenol had been completely removed. The contents of each of the cellophane bags were centrifuged separately to remove insoluble matter, the clear solutions were evaporated *in vacuo* to a small volume, frozen at $-10°$ and taken to dryness *in vacuo* from the frozen state.

Examination of unchanged antigenic material. The material (S.P.T. 12A) remaining insoluble after seven extractions of the original antigenic complex with 95% phenol was recovered by washing with alcohol to remove phenol, solution in water and reprecipitation with alcohol. The material was again dissolved in water and reprecipitated with alcohol. After drying *in vacuo*, the substance weighed 350 mg., contained 6·1% N, 1·5% P, no inorganic P and gave an opalescent solution in water. The quantitative Sakaguchi test indicated the presence of 0·7% arginine and the biuret, ninhydrin and diazobenzenesulphonic acid tests were strongly positive on 2 mg. samples of the preparation. These figures were very similar to those given by the original antigenic material and indicated that the part of the antigenic complex which had not dissolved in the 95% phenol remained essentially the same in composition as the original phospholipin-free antigenic complex. There was, therefore, no evidence that the protein component was simply admixed with the polysaccharide, but the results supported the belief that these substances were combined as a molecular complex. The material remaining insoluble in 95% phenol was tested for antigenicity in rabbits. The usual course of 3 doses of 0·05 mg. produced an immune response

Table 1. *Showing the production of 'Shiga' agglutinins in rabbits inoculated with substances derived from the phospholipin-free antigen by treatment with 95% phenol solution*

Material injected		Doses	Rabbit	Dilution of immune serum giving agglutination with formolized suspension of *Bact. dysenteriae* (Shiga)				
Description	Nature	mg.	no.	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320
Material insoluble in 95% phenol (S.P.T. 12A)	Polysaccharide-protein complex	3 × 0·05	231	4	3	1	1	0
			232	4	4	3	2	1
			233	4	2	1	0	0
			234	2	1	1	0	0
Material soluble in 95% phenol and soluble in water after dialysis (S.P.T. 16)	Polysaccharide	6 × 0·05	250	0	0	0	0	0
			251	0	0	0	0	0
			252	0	0	0	0	0
			253	0	0	0	0	0
Material soluble in 95% phenol and insoluble in water after dialysis (S.P.T. 13)	Protein	6 × 0·05	242	0	0	0	0	0
			243	0	0	0	0	0
			244	0	0	0	0	0
			245	0	0	0	0	0

4 Complete agglutination.
3 Almost complete agglutination.
2 Incomplete agglutination.
1 Trace of agglutination.
0 No agglutination.

in each of the four animals immunized (Table 1, animals 231, 232, 233 and 234). After a further course of immunization the sera of all rabbits gave precipitation when mixed with equal volume of 1 : 100,000 dilution of the 'Shiga' specific polysaccharide.

Isolation of the polysaccharide component. The results of an examination of the phenol-soluble fraction that was also soluble in water after dialysis are given in Table 2. It appears that the water-soluble material extracted by phenol from

Table 2. *Showing the amount and composition of material extracted from the antigen of* Bact. dysenteriae (*Shiga*) *by strong solutions of phenol*

Phenol extract	Volume ml.	Weight mg.	Nitrogen %	Total* phosphorus %	Arginine %
I	30	54·2	1·15	—	—
II	30	75·3	1·68	0·55	0·05–0·1
III	30	70·3	1·78	0·74	0·05–0·1
IV and V	2 × 20	86·4	2·27	1·00	0·10
VI	30	78·4	2·38	0·87	0·07
VII	30	67·0	2·10	0·98	0·01

* Inorganic P, 0·0%.

the antigenic complex was almost entirely polysaccharide in nature. Traces of arginine remained in the soluble material but this was to be expected in view of the very simple treatment involved. The combined extracts weighed 431·6 mg., and 5 mg. portions of the material failed to give a positive biuret, ninhydrin, α-nitroso-β-naphthol or diazobenzenesulphonic acid test. The Molisch test was strongly positive. Fractions II–VII after solution in water were combined, made distinctly acid with acetic acid; spun to remove a small amount of insoluble matter and precipitated by the addition of several volumes of alcohol. The preparation showed an opalescence which rendered the determination of specific rotation difficult. The substance contained 2·05% N, 0·81% P and no inorganic P. The material was tested in rabbits for antigenicity; 3 animals received 6 doses each of 0·05 mg. intravenously. There was slight production of specific agglutinins and precipitins although the immune response was negligible when compared with that elicited by the original phospholipin-free antigenic complex. The material was again dissolved in phenol solution (92%) and after the addition of 3% methyl alcohol, was dialysed in a cellophane bag until free from phenol. The solution was freed from a small amount of insoluble matter, concentrated to 10 ml. and taken to dryness *in vacuo*. A solution (1%) of the substance (S.P.T. 16) possessed slight opalescence, but the material (10 mg.) gave a negative Sakaguchi test. Arginine (0·01 mg.) under the same conditions gave a definite rose-pink coloration. Analytical data: $[\alpha]_{5461} + 84 \pm 3°$ (0·5% in H_2O); N, 1·82%; P, 0·75%, inorganic P, 0·0%. The material was again tested for antigenicity in 4 rabbits; there was no detectable immune response (Table 1, animals 250, 251, 252 and 253).

Isolation of the protein component. The material which separated during dialysis of the phenol extracts (I–VII) was collected and extracted with 2 ml. *N* NaOH. The insoluble residue was again extracted with 1 ml. 0·1 *N* NaOH and finally washed with several 1 ml. quantities of distilled water. The residue appeared to be largely inorganic, weighed 60 mg. and contained 0·38% N. The alkali-soluble material and the water washings were mixed together, acidified with acetic acid and the resulting precipitate was collected, dissolved by the addition of alkali to *p*H 8·0 and again recovered by the addition of acid. The

precipitate was thoroughly washed with 0·05*N* acetic acid, alcohol and dried *in vacuo*. The substance (S.P.T. 13) weighed 120 mg., gave an intense biuret test and according to an arginine determination made by Jorpes & Thorén's [1932] modification of Sakaguchi's test, contained about 5% of this amino-acid. The material gave an almost colourless solution (0·5% in 0·1*N* NaOH), possessed a strong negative rotation, $[\alpha]_{5461} - 60 \pm 3°$ (*c*, 1 in 0·1*N* NaOH), contained 12·3% N, no appreciable quantity of P, and a 5 mg. sample gave a very faint nitroprusside test for S after Na fusion. A diazobenzenesulphonic acid test on 1 mg. of the preparation gave a strong red coloration in 2–3 sec.; a control test employing the reagents alone gave a pale yellow colour only during the first 1–2 min. The fraction consists, therefore, of the protein that was originally associated with the polysaccharide that had been recovered in the phenol-soluble fractions I–VII. The results of antigenicity tests on this material are shown in Table 1 (animals 242, 243, 244 and 245); there was no detectable immune response. The figures given in Table 2 indicate that the total weight of the antigenic material dissolved in each 30 ml. of 95% phenol used for extraction remained approximately constant and that in all 530 mg. of the antigenic complex dissolved in 190 ml. of phenol solution.

An examination of the protein isolated by this method showed that the substance was more soluble in dilute acids than samples of protein prepared by acetic acid hydrolysis of the 'Shiga' antigen and that in this property the two protein preparations appeared to be different. For further investigation a quantity of the material was therefore prepared by the following simplified process. The diethyleneglycol primary extraction product (400 mg.) was dissolved in 90% phenol solution (20 ml.) and a small insoluble residue was removed. The clear solution was dialysed against running tap water in a cellophane bag until free from phenol and the precipitate which formed in the bag was collected. After extraction, first with alcohol and then with alcohol-ether mixture, to remove phospholipin, the material was dissolved in dilute NaOH and the small insoluble residue was removed by centrifuging. The substance was then subjected to three successive precipitations from dilute alkaline solution with acetic acid at *p*H 4·6, the protein thrown out of solution being redissolved each time in water by the addition of a minimum quantity of dilute NaOH. It was then noticed that the protein exhibited a marked solubility in *N* acetic acid and in HCl at about *p*H 2. Addition of further HCl, however, produced a heavy precipitate presumably due to the production of an insoluble hydrochloride. The protein was further purified by precipitating twice from an alkaline solution with HCl to make a final concentration of *N* HCl. The insoluble hydrochloride was washed with absolute alcohol to remove the excess of HCl, and the substance was then found to dissolve in distilled water to yield a clear colourless solution showing an acid reaction. The protein was finally precipitated from solution by the addition of sufficient solid sodium acetate to raise the *p*H to about 4·5. The protein component recovered in this manner was found to be free from P, showed $[\alpha]_{5461} - 74 \pm 2°$ (*c*, 1 in 0·1*N* NaOH) and contained 13·0% N. The protein could be precipitated from solution in *N* acetic acid by means of trichloroacetic acid, metaphosphoric acid, salicylsulphonic acid and other protein precipitants. Addition of common salt or other soluble chlorides, evoked the precipitation of the insoluble hydrochloride.

The analytical figures which we have previously given for the 'protein' component obtained from the diethyleneglycol primary extraction product by acid hydrolysis are significantly different from those obtained for the phenol-dissociated protein component. The former substance could be reprecipitated

many times from variable concentrations of the acid without appreciable loss, whereas the phenol-dissociated protein was readily soluble at about *p*H 2·5. It is apparent, therefore, that the proteins isolated from the same antigenic material by two different methods are substantially different in character and it appears that one product is most probably a degradation product of the other. The possibility that denaturation might be responsible for the difference was first considered, but the hypothesis appeared unlikely in view of the considerable difference in the analytical figures. Furthermore, it was found that the protein prepared by the phenol method, when dissolved to yield a clear solution in *N* acetic acid, could be heated for many hours at 95° without causing any precipitation to occur. The following experiment, however, indicates that the simple protein obtained by the phenol dissociation of the antigenic complex, is a derivative of the conjugated protein which is obtained by heating the antigenic complex with dilute acetic acid.

Isolation of the simple protein from the conjugated protein

A sample of the crude conjugated protein, prepared by hydrolysis of the antigenic complex with dilute acetic acid according to the usual method, was freed from traces of fatty impurities by extraction with an alcohol-ether mixture and was subsequently precipitated from faintly alkaline solution with acetic acid. The procedure was designed to limit the manipulations to the minimum consistent with the isolation of an apparently homogeneous material. The substance contained 11·2 % N, 1·07 % P, and possessed $[\alpha]_{5461} - 48 \pm 3°$ (*c*, in 0·1 *N* NaOH). The material was not soluble in *N* acetic acid, even on warming. The conjugated protein (400 mg.) thus obtained dissolved readily in 90 % phenol solution (10 ml.) and was largely thrown out of solution by the addition of ethyl alcohol (10 ml.). The precipitate was redissolved in phenol (10 ml.), again thrown out of solution by the addition of an equal volume of absolute alcohol, thoroughly washed with alcohol and finally dissolved in water by the addition of a few drops of NaOH solution. A small insoluble residue was removed and an equal volume of 2 *N* HCl was added to precipitate the protein from solution. The precipitate was washed with alcohol to remove the excess of acid. On addition of distilled water the precipitate slowly dissolved to a rather opalescent solution. The protein was again recovered by addition of sodium acetate to reduce the acidity of the solution to *p*H 4·5. After washing with alcohol the protein was taken up in 90 % phenol, a small fraction which remained undissolved was removed and the clear supernatant fluid was precipitated by addition of ethyl alcohol to 50 % concentration. The protein was once again precipitated from phenol solution by alcohol and again recovered from aqueous solution. The protein was then found to dissolve completely in *N* acetic acid on warming (35–40°) to yield a clear colourless solution. The protein was finally recovered as the insoluble hydrochloride. After washing with alcohol the hydrochloride yielded a clear solution of acid reaction on addition of distilled water, from which it was precipitated at *p*H 4·5 by the addition of sodium acetate. The material recovered (120 mg.) contained 13·8 % N, no P, and showed a specific rotation $[\alpha]_{5461} - 88 \pm 2°$ (1 % solution in 0·1 *N* NaOH). The analytical figures and general properties of the purified protein are distinctly different from those of the starting material, but show a close agreement with those of the substance prepared by phenol dissociation of the whole antigenic complex. It is not considered that either of the preparations isolated in this manner are pure but the simple amphoteric protein has been obtained in larger quantities by other methods and is described in more detail later in this paper.

Owing to the difficulty of establishing the purity of the conjugated protein it is not clear whether all the changes in the analytical data that accompany the changed solubility properties of the protein during the rather long series of manipulations described above can be correlated with the removal of a single chemical component that might be termed the prosthetic group. In previous purification procedures the conjugated protein was dissolved in a small volume of cold 0·1 *N* NaOH and reprecipitated with dilute HCl. This procedure was usually carried out five or six times and the substance was sometimes further purified by precipitation as sodium salt by the addition of alcohol to the alkaline solution of the material. Isolated in this manner, the protein preparations contained 11–12 % N and showed $[\alpha]_{5461}$ −45 to −50°. The P content fell steadily during purification and the final product usually contained not more than 0·2 % P. From these observations it appears that the P of the protein component obtained by acetic acid hydrolysis of the antigenic complex can be largely removed without causing a significant increase in the specific rotation or nitrogen content of the protein substance. On the other hand repeated phenol treatment of the protein component of the antigen liberated by acetic acid hydrolysis not only removes completely any P present but also causes the resulting protein to possess a higher laevorotation (−82 ± 5°), a higher N content (13·5–14·0 %) and to show a changed solubility at *p*H 2·0–2·5. The influence of these changes on the immunological properties of the preparations will be described later in the paper.

Extraction of Bact. dysenteriae (*Shiga*) *with 90 % phenol solution*

In view of the marked solubility of the 'Shiga' antigenic material in 90 % phenol solution, and the observed insolubility of agar and of nucleic acid in this solvent, it was decided to attempt a phenol extraction, in the first instance, of a sample of organisms that had already been repeatedly extracted with diethyleneglycol, but which contained a further quantity of antigenic material. Organisms that had already been in contact with anhydrous diethyleneglycol at 0–2° for many months and which had been repeatedly extracted with fresh diethyleneglycol and had yielded about 7 % of their dry weight as antigenic material were observed to have remained morphologically intact.

The heavy paste of organisms collected from the bowl of the Sharples supercentrifuge after removing the excess of diethyleneglycol was repeatedly triturated with acetone, washed with this solvent and dried *in vacuo*. The fine white powder (132 g.) so obtained was shaken with 90 % phenol solution (800 ml.) and after standing for 96 hr. with occasional shaking, the bacterial residue was again collected in a Sharples centrifuge. The clear supernatant was passed through a Doulton filter candle and mixed with 3 vol. of alcohol. The precipitate which formed was then suspended in water and dialysed against water in a cellophane bag until free from phenol. The bag finally contained a heavy white precipitate and a strongly opalescent supernatant solution. The precipitate was removed by centrifuging, dried after washing with water and alcohol and weighed. The supernatant was evaporated to dryness *in vacuo* from the frozen state. Table 4 shows the yields obtained with six successive extractions of the bacteria with 90 % phenol. As the extraction proceeded the organisms disintegrated until finally the bacterial residue appeared as a clear gelatinous mass.

The material soluble in water

The white fibrous material prepared by drying the supernatant fluid from the dialysis bags, dissolved readily in water to yield heavily opalescent solutions. The extracts were pooled, suspended in water to yield a 1 % solution and the

Table 3. *Showing the production of 'Shiga' agglutinins and precipitins in rabbits inoculated with substances isolated from the antigen of* Bact. dysenteriae (*Shiga*)

Material used as antigen	No. of doses	Total antigen injected mg.	Animal no.	Precipitation test: Dilutions of polysaccharide 1 : 10^4	1 : 10^5	1 : 10^6	Agglutination test*: Dilutions of immune serum 1 : 10	1 : 20	1 : 50	1 : 100	1 : 200	1 : 500
Phenol-extracted antigen	3	0·15	228	4	4	1	4	4	4	4	4	0
			229	3	2	1	4	4	3	2	0	0
	3	0·08	253	4	4	2	4	4	4	4	4	3
			256	4	4	2	4	4	4	4	4	3
Undegraded polysaccharide prepared by phenol method (preparation S 27)	6	0·30	238	0†	0	0	0	0	0	0	—	—
			239				0	0	0	0	—	—
			240				0	0	0	0	—	—
			241				0	0	0	0	—	—
Undegraded polysaccharide prepared by alkali method (preparation S 56)	6	0·30	200	0†	0	0	0	0	0	0	—	—
			201				0	0	0	0	—	—
			202				0	0	0	0	—	—
			203				0	0	0	0	—	—
'Shiga' conjugated protein	3	0·15	243	0	0	0	0	0	0	0	—	—
			248	0	0	0	0	0	0	0	—	—
			257	0	0	0	0	0	0	0	—	—
			260	0	0	0	0	0	0	0	—	—
			261	0	0	0	0	0	0	0	—	—
Conjugated protein combined with polysaccharide S 27	3	0·08	237	3	4	1	4	4	4	4	4	3
			238	3	4	1	4	4	4	4	4	3
			239	3	4	1	4	4	4	4	4	3
Conjugated protein combined with polysaccharide S 56	3	0·08	249	1	3	1	4	4	4	4	2	0
			250	1	2	1	4	4	4	4	3	1
			251	0	1	1	4	4	3	2	0	0
Electrophoretically pure 'Shiga' simple protein combined with polysaccharide S 50	3	0·08	257	0	0	0	0	0	0	0	—	—
			260	0	0	0	0	0	0	0	—	—
			261	0	0	0	0	0	0	0	—	—

* Formolized whole culture of 'smooth' *Bact. dysenteriae* (Shiga).
† A pooled sample only was tested.

Table 4. *Material extractable with 90 % phenol from 'smooth'* Bact. dysenteriae (*Shiga*)*

Extract no.	Volume of extract ml.	Water-soluble material g.	Water-insoluble material g.
1	315	3·10	3·50
2	320	2·07	2·99
3	324	1·10	2·98
4	372	0·14	1·20
5	344	0·08	0·88
6	410	—	0·46
Total weight of extract		6·49	12·01
Percentage of dry bacteria		4·9	9·1

* After previous treatment with diethyleneglycol [see Morgan, 1937].

material was precipitated with acetone. The substance thrown out of solution between the levels 50 and 65 % acetone was collected, redissolved in water and again precipitated with acetone. The material obtained in this manner represented the bulk of the substance present in the supernatant fluid from the dialysis bags. The purified material was tested in rabbits and was found to be a powerful 'Shiga' antigen. Three doses of the substance, when given intravenously at intervals of 3 days, yield strongly agglutinating sera (Table 3, animals 228, 229, 253 and 256). The antigenic substance contained 5·8 % N, and 0·80 % P. In order to show that this material was similar to the phospholipin-free diethyleneglycol primary extraction product the following experiments were carried out.

Hydrolysis with 1 % acetic acid. The antigenic material (1 g.) was dissolved in water (66 ml.) and glacial acetic acid (0·66 ml.) added. The solution was treated in exactly the same manner as for the acid hydrolysis of the primary extraction product [Morgan & Partridge, 1940, 1]. The water-insoluble precipitate (293 mg.) after extraction with ether gave an almost clear solution in dilute alkali and proved to be insoluble at all concentrations of acetic acid to 2*N*. After precipitation from slightly alkaline solution (*p*H 8·0) by addition of acetic acid the material contained 10·8 % N, 0·8 % P and showed $[\alpha]_{5461} - 55 \pm 5°$. The substance was further purified by solution in ice-cold 0·1*N* NaOH to make a 1 % solution and precipitated by the addition of alcohol to yield 80 % by vol. and a few drops of acetic acid. The substance then contained 11·2 % N and 0·65 % P, and showed $[\alpha]_{5461} - 56 \pm 5°$. The analytical figures are in good agreement with those obtained for the conjugated protein isolated in a similar manner from the diethyleneglycol primary extraction product. The substance was tested in animals for antigenicity and was found to behave like the so-called 'polypeptide' previously isolated [Partridge & Morgan, 1940]; specific *Bact. dysenteriae* (Shiga) precipitins and agglutinins were not produced but the formation of homologous precipitins was observed.

The clear supernatant fluid from the hydrolysis mixture after removal of the water-insoluble protein component was evaporated under reduced pressure to a small volume and precipitated by addition of 15 vol. of absolute alcohol to which a few crystals of sodium acetate were added to ensure complete precipitation. The material obtained weighed 505 mg. Specific precipitation with *Bact. dysenteriae* (Shiga) immune rabbit serum was given by dilutions of the substance out to $1 : 10^6$. The material gave a colour which matched closely in tint and intensity that produced by 'Shiga' specific polysaccharide when the two were compared according to the orcinol method of Sørensen & Haugaard [1933]. After

purification in the usual manner [Morgan, 1936] the polysaccharide contained 1·68 % N and showed $[\alpha]_{5461}+106\pm2°$.

Dissociation in phenol solution. The antigenic material (1 g.) was dissolved in 90 % phenol solution (33 ml.) and after removing a small residue was dialysed against frequent changes of distilled water. A heavy precipitate of material insoluble in water gradually formed during the removal of the phenol. Precipitation of the substance was complete when the phenol had been entirely eliminated. The contents of the bag were then centrifuged, the insoluble material collected, dissolved in dilute alkali and precipitated by addition of acetic acid to *p*H 4·6. The fraction weighed 290 mg. The substance formed a clear solution in *N* acetic acid and in HCl at *p*H 2·0–2·5 and thus behaved like the protein obtained in this manner from the diethyleneglycol primary extraction product. The addition of further HCl, or a soluble chloride precipitated the protein as hydrochloride. The protein contained 14·5 % N and P was absent; $[\alpha]_{5461}-78\pm5°$. The somewhat opalescent supernatant from the dialysis bag was concentrated and precipitated by addition of 4 vol. of ethyl alcohol in the presence of a little sodium acetate. The precipitate (559 mg.) proved to be largely undegraded 'Shiga' polysaccharide. The material contained 2·1 % N and 1·33 % P. The viscosity of a 0·5 % solution in 0·9 % NaCl solution was 1·32 (solvent, 1).

The above experiments show the antigenic material isolated by phenol extraction of the organism to be similar in chemical constitution and physical properties to the diethyleneglycol primary extraction product except for the expected absence of the phospholipin component.

The material insoluble in water

From the yield of this fraction obtained by phenol extraction and subsequent dialysis (Table 4) it will be seen that it represents a major constituent of the somatic substance of *Bact. dysenteriae* (Shiga). The substance dissolved, except for a small residue, in dilute NaOH, showed all the usual qualitative tests for proteins and was precipitated quantitatively from alkaline solution by addition of acetic acid to *p*H 4·6.

A sample of the protein (2·5 g.) was dissolved in dilute NaOH (40 ml.) and a fraction (0·59 g.) removed at 75 % alcohol level. This material was set on one side to be worked up later with a further batch of material. A second precipitate was obtained by increasing the alcohol concentration to 80 %. A small third fraction was recovered by neutralizing the 80 % alcoholic supernatant fluid with acetic acid. It was observed that the original substance showed a considerable solubility in 90 % alcohol in the presence of alkali or of excess acid, especially when salts were present. The second and third fractions were mixed, dissolved in a few ml. dilute NaOH and yielded a perfectly clear, colourless solution. The protein was finally precipitated as the hydrochloride by the addition of HCl to *N* concentration. The precipitate dissolved in distilled water, presumably owing to hydrolysis of the acid salt, and the addition of solid sodium acetate to reduce the acidity of the solution to about *p*H 4 caused the material to precipitate completely. The substance (1 g.) was washed several times with alcohol and dried *in vacuo*. The purified protein was free from P and contained 13·7 % N (uncorrected for ash). Specific rotation $[\alpha]_{5461}-81\pm2°$ (in 0·1 *N* NaOH); $-79\pm2°$ (in *N* acetic acid). The protein developed no colour with Sørensen & Haugaard's orcinol reagent for the detection of carbohydrate.

Electrophoresis. A sample of the protein was studied in the Tiselius electrophoresis apparatus through the kindness of Dr R. A. Kekwick. The protein was dissolved to make a 2 % solution in phosphate buffer of *p*H 8, μ 0·1, containing

a drop of dilute NaOH and dialysed against the buffer solution at 0° and at constant volume until equilibrium was attained. The solution was then clear and colourless. The Schlieren picture showed the substance to be electrophoretically homogeneous.

Phenol extraction of a 'rough' strain of Bact. dysenteriae (*Shiga*)

The rough organisms, which remained as a glycol-moist paste (83 g.) after a diethyleneglycol extraction experiment, were taken up in 90 % phenol and treated in the same way as for the 'smooth' organisms. The clear phenol extract after dialysis against distilled water to remove phenol contained a heavy deposit of water-insoluble material. The supernatant solution after the precipitate was removed was quite clear and on evaporation to dryness was found to be practically free from dissolved material, thus confirming the absence of the specific antigen from this variant of the organism. The insoluble material appeared to be essentially protein in nature and after purification it dissolved readily to form clear solutions in dilute NaOH and also in *N* acetic acid. The material contained 13·5 % N (uncorrected for ash), no P and showed $[\alpha]_{5461} - 80°$ (*c*, 0·5). The substance appeared to be identical with the phenol-soluble amphoteric protein of the 'smooth' organism.

Dissociation of the antigenic complex by means of alkali

The amino-acid-containing component can readily be removed from the phospholipin-free antigenic complex by repeated precipitation of the material from alkaline solution by alcohol. It is essential that during the process the solution is kept as near 0° as possible since it has been found that on standing at room temperature or 37° alkaline solutions of the undegraded polysaccharide tend to lose their high viscosity and give rise to a degraded form of the specific polysaccharide. In one experiment a preparation (0·75 g.) of phospholipin-free antigenic material ($[\alpha]_{5461} + 65 \pm 5°$; N, 4·4 %) was dissolved in distilled water (75 ml.) cooled to 0° and NaOH cautiously added to make a 0·05*N* solution. Cold alcohol (300 ml.) was then added slowly with vigorous shaking and the solution was left at 0° for an hour. The precipitate was collected, thoroughly dialysed at 0° and the resulting solution dried *in vacuo* from the frozen state. The preparation showed $[\alpha]_{5461} + 79 \pm 3°$; N, 3·5 %. The alkaline alcoholic supernatant was acidified with acetic acid, concentrated *in vacuo*, dialysed and taken to dryness. The material obtained showed a negative rotation and gave a strong biuret test for protein. It was not further purified but was put aside to be mixed with similar fractions obtained from the subsequent alkaline supernatant fluids.

The main fraction that was precipitated by 80 % alcohol was again dissolved in water at 0° and the alkaline precipitation process was repeated. The N content of the material (0·43 g.) again fell and the specific rotation increased to $+82 \pm 5°$. A further alkaline treatment gave a product showing $[\alpha]_{5461} + 84 \pm 5°$; N, 2·2 %; P, 0·56 %. Arginine determined by Sakaguchi's method was 0·05 % and the biuret test was negative on 10 mg. of the substance. Two further precipitations from alkaline solution by alcohol gave a product that showed $[\alpha]_{5461} + 84 \pm 2°$; N, 2·07 %; P, 0·57 %. The material dissolved in water to give a clear viscous solution. A 0·5 % solution in 0·9 % NaCl gave a viscosity at 37° of 1·96 compared with the solvent as unity (Ostwald viscosimeter). The substance was given intravenously to a group of eight rabbits. Six doses of 0·05 mg. at 2–3 day intervals gave rise to no demonstrable 'Shiga' agglutinins or precipitins in any of the animals immunized whereas the original phospholipin-free complex before

alkaline treatment induced the formation of high titre agglutinins and precipitins when given in similar doses. The results obtained with four only of the rabbits immunized are shown in Table 3 (animals 200, 201, 202 and 203).

The protein component recovered by acidifying the alkaline alcoholic supernatant liquids was freed from admixed polysaccharide by repeated precipitations from alkaline solution by the addition of acetic acid to *p*H 4·5. On warming gently, the precipitate dissolved to yield a clear, colourless solution in *N* acetic acid. The protein was recovered from the acetic acid solution by the addition of NaOH to bring the solution to *p*H 4·5, and, after washing the precipitated protein thoroughly with alcohol to remove sodium acetate, was found to contain 13·8 % N. The substance was free from P and showed $[\alpha]_{5461} - 74 \pm 4°$. The analytical figures are similar to those for the simple protein prepared by phenol dissociation of the antigenic complex and indicate that the protein component of the antigen is obtained as a result of dissociation in alkaline alcoholic solution, largely in the amphoteric form.

A preparation of antigenic material that had been obtained by phenol extraction of a dry culture of *Bact. dysenteriae* (Shiga) was separated into its components by fractionation from alkaline solution as described above. The material (2 g.) was dissolved in 100 ml. of ice-cold water and made *N* with NaOH. Alcohol (200 ml.) at $-10°$ was then slowly added with vigorous shaking, the solution was allowed to stand at 0° for about 30 min., and the precipitate was collected and redissolved in 100 ml. of cold water. The process of precipitation from alkaline solution was repeated twice, the combined alkaline-alcoholic solutions being acidified with acetic acid and the protein component, which was thrown out of solution, was recovered in the usual manner. The polysaccharide finally isolated analysed as follows: $[\alpha]_{5461} + 85 \pm 2°$ (*c*, 0·5); N, 2·01 %; P, 0·55 %, inorganic P, nil. The viscosity of a 0·5 % solution in 0·85 NaCl was 1·36 relative to 0·85 % saline as 1 (Ostwald viscosimeter). The protein component showed $[\alpha]_{5461} - 80 \pm 3°$ (*c*, 0·5 % in 0·1*N* NaOH); N, 13·9 %; P, nil; and arginine estimated by Sakaguchi's method as modified by Jorpes & Thorén [1932], 6·8 %. The analytical figures obtained show that the antigenic material extracted from *Bact. dysenteriae* (Shiga) by phenol contains the same polysaccharide and protein components as the antigenic complex extracted from the organisms by diethyleneglycol.

In a series of experiments the reaction conditions were varied in order that some estimate might be made of the most suitable conditions to effect the maximum dissociation. A preparation of phospholipin-free antigenic material was dissolved in water and NaOH added to a final concentration of 0·1 *N*. After standing at 20° for 1 hr. the solution was treated with alcohol to make a final concentration of 75 % by vol. The precipitate, which contained most of the polysaccharide present, represented 77 % of the material used and contained 4·1 % N. The protein recovered from the 75 % alcoholic solution contained 11·5 % N and represented 12·5 % of the complex. The experiment was then repeated, but alcohol was added immediately after the addition of the alkali, so that the latter was only in contact with the complex for 10–15 sec. The distribution of the polysaccharide and protein components was found not to be significantly different from that observed in the previous experiment. The material precipitated by 75 % alcohol represented about 75 % of the complex and contained 3·7 % N while the crude protein recovered from the supernatant contained 10·9 % N and represented 20 % of the starting material. From these results it appears that the separation of the components from an alkaline solution of the antigenic material is a process that depends upon dissociation of the complex at high *p*H values and is not due to alkaline hydrolysis which would presumably depend largely upon

the time during which the alkali is in contact with the complex. In all experiments, repeated fractionation from alkaline solution has been necessary to produce a sample of polysaccharide uncontaminated with protein.

An experiment was also undertaken in order to form a rough estimate of the minimum conditions of alkalinity required to produce a useful degree of dissociation. The antigenic material was made up to a concentration of 1% in (*a*) 10% potassium acetate solution, and (*b*) 0·1 *N* ammonia solution (*p*H 11·0) and after a few minutes alcohol was added to make a final concentration of 75% by vol. The thoroughly washed precipitates contained 5·5% and 5·3% N respectively and in each instance practically the whole of the material was recovered as precipitate. Acidification of the alcoholic filtrate yielded no appreciable precipitate of protein. It appears, therefore, that strong alkali is essential in order to induce a degree of dissociation sufficiently marked to be of use for the purpose of separating the constituents by alcoholic fractionation.

The formation of complexes between the specific polysaccharide of Bact. dysenteriae (*Shiga*) *and proteins*

It has already been mentioned that degradation of the 'Shiga' polysaccharide takes place readily in the presence of strong alkali at 37°, or in neutral or acid solution at higher temperatures, the result being a reduction in the viscosity of the aqueous solution, and it was subsequently found that the degraded polysaccharide no longer possesses the capacity to form soluble complexes with proteins.

The undegraded specific polysaccharide, on the other hand, prepared by phenol or alkali dissociation of the antigenic substance forms acid-soluble complexes with proteins that are themselves, like the 'Shiga' protein component, normally insoluble in aqueous solution at acid reaction. If a solution of the specific polysaccharide and an alkaline solution of a protein are mixed and acidified with acetic acid a soluble, stable complex is formed and the protein is found to be no longer precipitated by the addition of trichloroacetic acid or the other acid protein precipitants, provided that an excess of the protein above that found in the native antigenic complex has not been used.

In one experiment a number of complexes of various proteins with the undegraded polysaccharide prepared by phenol dissociation of the antigenic substance were formed as follows. The protein, 0·5 ml. of a 1% solution in 0·1 *N* NaOH, was added to 2 ml. of an 0·5% solution of the specific polysaccharide. The mixture was kept for 5–10 min. at 25–30° and acidified to *p*H 4·5 by cautious addition of 0·1 *N* acetic acid. The proteins employed were hen's egg vitellin, stromatin, prepared from human erythrocytes according to the method of Jorpes [1932], electrophoretically pure human serum-γ-globulin, 'Shiga' conjugated protein and the simple protein prepared from it by phenol treatment. In each instance the solution developed a bluish opalescence on acidification, but no precipitate was formed on the addition of 10% trichloroacetic acid solution (2 ml.). Control experiments were made in which the 'Shiga' polysaccharide was replaced by gum acacia, gum tragacanth and a sample of degraded (low viscosity) 'Shiga' polysaccharide, but in each instance a heavy precipitate of protein appeared on acidification of the mixed protein-polysaccharide solution with acetic acid or trichloroacetic acid. If the protein content of the alkaline mixture of the undegraded specific polysaccharide and any of the proteins mentioned was raised above 30% a considerable opalescence developed upon acidification, while a large protein excess in the mixture gave rise to a precipitate on addition of trichloroacetic acid.

Although the complexes formed by the undegraded polysaccharide with all the proteins investigated are alike in their behaviour with protein precipitants and in the appearance of their aqueous solutions, a considerable variation in their stability toward heat treatment was noticed. The complex formed from the polysaccharide and 'Shiga' conjugated protein behaves, on heating in acetate buffer, in a similar way to the antigenic diethyleneglycol primary extraction product [Morgan & Partridge, 1940, 1], that is to say, it withstands heating to 100° at *p*H 4·6 for 2–3 hr. before flocculation occurs and the conjugated protein is thrown out of solution. Under these experimental conditions the polysaccharide itself becomes degraded and loses its power to associate with the protein. On the other hand, the complexes formed with the other proteins mentioned, including the simple amphoteric protein derived from the 'Shiga' antigen or from the organism itself, are heat-labile. On raising the temperature of a solution of the complexes in acetate buffer at *p*H 4·6 by immersion in a boiling water bath, flocculation commences almost at once and is complete in 4–5 min., the protein separating as an insoluble precipitate from the clear supernatant fluid. These experiments indicate that whereas the complex formed by recombination of the 'Shiga' polysaccharide and the conjugated protein is similar in constitution and physical properties to the antigenic substance obtained by extraction of the micro-organism with diethyleneglycol, the complex formed by the polysaccharide with other proteins cannot be regarded as a true reversal of the dissociation of the 'Shiga' antigen in alkaline solution. It is to be noted that the production of the latter type of complex indicates the danger of placing too much reliance on the use of the usual acid protein precipitants to indicate the absence of protein from material containing a large percentage of undegraded bacterial polysaccharide.

Antigenicity of the artificial complexes

The complex formed by acidifying an alkaline solution of purified, undegraded polysaccharide and the 'Shiga' conjugated protein is a powerful antigen when tested in rabbits. In one experiment the complex was formed as follows: A sample (25 mg.) of the conjugated protein (11·3 % N, 0·58 % P, $[\alpha]_{5461} - 52 \pm 2°$) was dissolved in 2·5 ml. of 0·1 *N* NaOH and added to 7·5 ml. of a 1 % solution of the undegraded 'Shiga' polysaccharide prepared by the phenol method. The mixture was kept 5 min. at 25° and then cautiously acidified to about *p*H 4 by addition of acetic acid. The solution, which had a faint bluish opalescence, was evaporated to dryness *in vacuo* from the frozen state. Three doses of the artificial complex, amounting to 0·08 mg., were sufficient to produce strongly precipitating sera which also agglutinated killed suspensions of *Bact. dysenteriae* (Shiga) to about the same dilution as sera prepared against similar doses of the diethyleneglycol primary extraction product (Table 3, animals 237, 238 and 239). The samples of conjugated protein and undegraded polysaccharide used were both tested in rabbits under similar conditions and were found to be inactive (Table 3, rabbits 243, 248, 257, 260, 261 and 238, 239, 240, 241 respectively).

The same sample of polysaccharide (S 27) was combined in a similar way with the proteins, vitellin, stromatin, gelatin and serum-γ-globulin, together with the simple protein prepared by phenol dissociation of the antigenic complex and a sample of protein prepared from a 'rough' strain of *Bact. dysenteriae* (Shiga) which is described later in this paper. Two rabbits were used for each of the preparations and the immunization was continued for a total of six doses, each of 0·05 mg. In no case was any 'Shiga' precipitin or agglutinin formation

observed. Similar unsuccessful attempts to produce artificial antigens by combining the undegraded polysaccharide with such proteins as casein and denatured rabbit serum protein have already been reported [Partridge & Morgan, 1940]. On the other hand, the conjugated protein obtained from the 'Shiga' organism, and a similar substance obtained from the 'O' antigen of *Bact. typhosum* both yield polysaccharide-specific antigens on combination with a number of polysaccharides of vegetable and animal origin [Morgan & Partridge, 1940, 2; Morgan, 1941 and unpublished work]. These results, taken together, lend support to the view that in antigen formation a specific grouping in the conjugated protein is involved and that this grouping is not contained in the other protein substances examined.

The ultra-violet absorption spectrum of the amphoteric protein and the conjugated protein

The ultra-violet absorption spectra of a number of the bacterial protein preparations were determined for us by Dr R. A. Morton, to whom we express our thanks. In Fig. 1 the spectrum of a sample of the simple protein prepared by

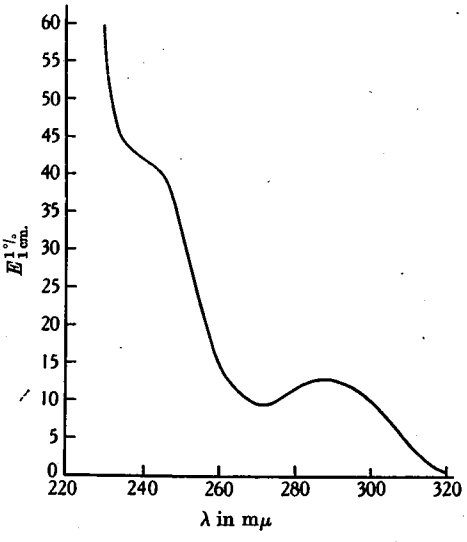

Fig. 1. Ultra-violet absorption spectrum of simple protein in 0·1 *N* NaOH.

phenol dissociation of the diethyleneglycol primary extraction product and dissolved in 0·1 *N* NaOH is recorded and is of the general type expected for substances of protein or peptide nature. The absorption at 280–290 mμ is normal for a protein containing tyrosine [Stenström & Reinhard, 1925, 1, 2], but the significance of the absorption at 235–245 mμ is not understood. Spiegel-Adolf & Seibert [1934] have shown that the pseudoglobulin in alkaline solution absorbs light selectively between 276 and 289 mμ whereas various nucleic acid preparations and their constituent pyrimidines under similar conditions absorb within the range 240–276 mμ with maximum absorption at about 265 mμ. Rough estimates of the tyrosine and tryptophan contents of the protein preparations examined have been made from the absorption data according to the method of Holiday [1936].

Using the equations given by Holiday,

$$M_1 \text{(tyrosine)} = (1{\cdot}0\ E_{305} - 0{\cdot}092\ E_{280}) \times 10^{-3},$$

the tyrosine content of one particular sample of amphoteric protein was calculated as 7·2 %. An estimate of the tryptophan content was also derived from the equation

$$M \text{(tryptophan)} = (0{\cdot}21\ E_{280} - 0{\cdot}288\ E_{305}) \times 10^{-3},$$

and led to a tryptophan value of about 2 %. In an earlier paper the tryptophan content determined by a colorimetric method was incorrectly given as 6 %. The absorption spectrum of a sample of simple protein derived from a 'rough' variant of *Bact. dysenteriae* (Shiga) by means of phenol extraction was almost identical with that obtained for the protein substance prepared in a similar manner from a 'smooth' strain of the organism.

The ultra-violet absorption spectra of several samples of the conjugated protein were also examined and were found to be very similar to those of the simple amphoteric protein. A strong absorption at 280–290 mμ indicated the presence in the molecule of approximately the same amount of tyrosine. The intensity of the absorption within the range 230–250 mμ, however, varied considerably with different preparations of the conjugated protein, but most specimens examined gave more intense absorption within this range than preparations of the simple amphoteric protein. It has also been observed that preparations of conjugated protein contain a yellow pigment and it is not yet known whether the prosthetic group of the conjugated protein is to be identified with this chromophoric substance or with the absorption within the range 230–250 mμ. Further work on this aspect of the problem is being undertaken.

Serological comparison of the 'Shiga' proteins

It has already been demonstrated that the conjugated protein gives rise to homologous precipitins when injected into rabbits in small doses by the intravenous route [Partridge & Morgan, 1940]. Stronger sera than those previously prepared have now been obtained by giving larger doses of the protein and by limiting the operations involved in its purification. The more recent samples of conjugated protein have been prepared with the minimum of manipulation necessary to free the material from contaminating specific polysaccharide and are characterized by a higher phosphorus content. Table 5 shows the result of immunizing a group of four rabbits with a sample of conjugated protein that contained 11·3 % N, 0·58 % P and showed $[\alpha]_{5461} - 52 \pm 2°$. Further groups of rabbits were also immunized with samples of the electrophoretically pure simple protein prepared from the 'smooth' organism, and with the simple protein prepared from the 'rough' strain. When immune sera collected after the first course of antigen are employed, it appears that the anti-conjugated protein serum reacts with the homologous antigen only, whereas, after a further course of injections the strength of the conjugated protein antiserum is considerably increased and its specificity is spread so that it then reacts with the preparations of simple protein. The amphoteric proteins themselves appear to be relatively poor antigens, and the sample prepared from the 'rough' strain of *Bact. dysenteriae* (Shiga) alone engendered the formation of weak homologous precipitins. On the other hand, the antiserum to the 'rough' protein does not react with the conjugated protein or with the simple protein from the corresponding 'smooth' strain. For brevity the results obtained with pooled sera are given in Table 5, but each serum was also tested separately, and each gave similar results to those of the corresponding pooled serum samples. A sample of antitoxin prepared in

Table 5. *Precipitation reactions with proteins isolated from* Bact. dysenteriae (*Shiga*)

Serum prepared against	Immunization doses mg.	Dilutions of test antigen: Conjugated protein $1:10^3$	$1:10^4$	$1:10^5$	$1:10^6$	Simple protein from 'smooth' strain $1:10^3$	$1:10^4$	$1:10^5$	$1:10^6$	Simple protein from 'rough' strain $1:10^3$	$1:10^4$	$1:10^5$	$1:10^6$
Conjugated protein (pooled antisera from 4 rabbits)	1st course $3 \times 0{\cdot}2$	2	1	1	0	0	0	0	0	0	0	0	0
	2nd course 3×1	3	4	3	2	2	1	0	0	3	3	1	0
	3rd course 3×1	4	4	3	2	2	1	0	0	4	3	2	1
Simple protein from 'smooth' strain (pooled antisera from 3 rabbits)	1st course $6 \times 0{\cdot}05$	0	0	0	0	0	0	0	0	0	0	0	0
Simple protein from 'rough' strain (pooled antisera from 2 rabbits)	1st course $3 \times 0{\cdot}2$	—	—	—	—	0	0	0	0	0	0	0	—
	2nd course 3×1	0	0	0	0	0	0	0	0	2	1	0	0
	3rd course 3×1	1	0	0	0	0	0	0	0	2	2	1	0

the rabbit against the toxic filtrates of the 'rough' organism [Neisser & Shiga, 1903; Conradi, 1903; Todd, 1904] gave no reaction with the test-antigens used in Table 5. A sample of toxic protein prepared by the trichloroacetic acid method of Boivin & Mesrobeanu [1937; Mesrobeanu & Boivin, 1937] was also tested against the three types of antisera given in Table 5 but no significant precipitation was observed. From these results it seems that the prosthetic group plays some role in determining the specificity of the conjugated protein.

Discussion

As a result of our earlier investigations on the chemical nature of the specific antigen of *Bact. dysenteriae* (Shiga) evidence was obtained for the view that antigenic substances prepared from *Bact. dysenteriae* (Shiga) by various methods owe their activity to the presence of two major components, a specific polysaccharide and an acidic substance that contains amino-acids and is largely polypeptide or protein in nature. Other components undoubtedly occur in the native antigen but it was found that these could be eliminated if suitable means were employed, without seriously impairing the antigenic qualities of the resulting material. The removal of these components, however, caused an alteration in the chemical and physical properties of the native antigenic complex. A method was described for the stepwise degradation of the antigenic complex which was based on the dissociating action of formamide. The non-antigenic specific polysaccharide isolated by this means could be recombined with the polypeptide-like component, obtained by acid hydrolysis of the native antigenic complex, to yield a powerful antigen that induced in rabbits the formation of immune-body specific for *Bact. dysenteriae* (Shiga).

The polypeptide-like component previously isolated from the antigenic substance by hydrolysis with dilute acetic acid at 100° contains 11·5–12·5% N and 0·6–8% P, and is readily soluble in dilute alkali but is insoluble throughout the entire acid *p*H range. An extensive amino-acid analysis of the material was not attempted, but it was shown to contain tyrosine, arginine, glutamic acid and tryptophan. The substance is digested by pepsin and by trypsin and, after acid hydrolysis, about half the total nitrogen appears as free amino-nitrogen. The low nitrogen content of the material, the poor yield of α-amino groups after acid hydrolysis and its unusual insolubility under acid conditions were considered sufficient reason for rejecting its classification as a simple protein without further investigation, and the material was at that time therefore described as a 'polypeptide-like' substance.

The results of the experiments that are described in the present paper indicate that the phospholipin-free antigenic complex can also be dissociated into its two major components by solution in 90% phenol. The addition of alcohol to the phenol solution causes the liberated polysaccharide and protein molecules to recombine and the resulting complex that is thrown out of solution is found to be antigenic. Direct dialysis of the phenol solution of the antigenic complex, however, enables the separated components to be readily isolated. The polysaccharide and protein constituents obtained by this means are unable to induce the formation of 'Shiga' agglutinins and precipitins.

In earlier attempts to purify the 'polypeptide', it was repeatedly dissolved in dilute alkali and reprecipitated by addition of acid and it was found that during this process the phosphorus content was slowly reduced while the specific rotation and nitrogen content of the substance showed only slight increases, but there was some indication that during the process the capacity of the substance to recombine with 'Shiga' polysaccharide to form an antigenic complex was

reduced, although no conclusive evidence on this point has yet been obtained. The adoption of the new technique for the dissociation of the antigenic complex in strong solutions of phenol reveals that the protein component recovered possesses markedly different properties from those of the 'polypeptide-like' substance isolated by acid hydrolysis of the native antigen. The nitrogen content approaches 14·5%, phosphorus is entirely absent and the specific rotation is considerably more *laevo*. Furthermore, the protein isolated by phenol dissociation is readily soluble in aqueous solution at *p*H 2·0–2·5 although it is insoluble at lower *p*H values than 2·0 and at higher values up to *p*H 7·0. The action of phenol on the polypeptide component itself shows that after several treatments with this solvent the bulk of the substance can be recovered as a material possessing properties very similar to, if not identical with, those of the simple protein isolated by the action of phenol on the whole antigenic complex. It becomes evident, therefore, that the polypeptide component is composed of the simple amphoteric protein and an additional substance (or substances) responsible for its acidic nature and its low nitrogen and high phosphorus contents. In the light of these observations it is suggested that the polypeptide-like component is a conjugated protein. The relationships of the different degradation products derived from the antigenic complex are set out in Fig. 2.

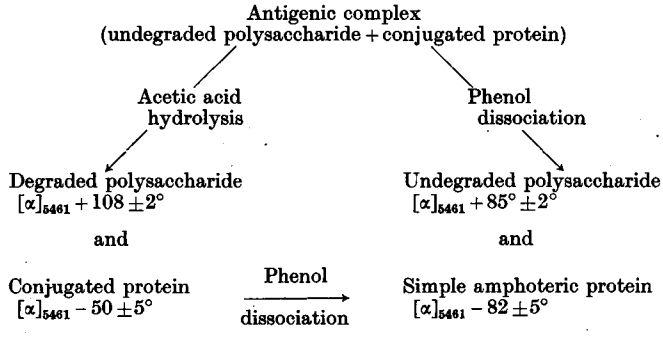

Fig. 2

It would be of considerable interest to know whether the phenol-soluble amphoteric protein which is present in both the 'rough' and 'smooth' forms of the organism is the precursor of the more complex conjugated protein component of the specific bacterial antigen and whether the development of this antigen as an additional component in the 'smooth' variant depends on enzymic mechanisms that synthesize the specific polysaccharide and convert the simple amphoteric protein into the conjugated and more reactive protein. Evidence has already been given [Partridge & Morgan, 1940] and is again demonstrated in the present paper, that under almost physiological conditions—aqueous isotonic saline solution at *p*H 8—these two components will, if present together, enter into combination and give rise to an antigenic complex. The nature of the bonds which hold the components together is unknown. It can only be stated that the forces concerned give rise to an aggregate with sufficient stability to make it behave as an independent molecular species. Furthermore, since the protein component, although essential for the manifestation of antigenicity, plays but a minor role in the determination of the immunological specificity of the antigen it would not be surprising to find a similar conjugated protein present in the specific somatic antigen of other gram-negative micro-organisms which

possess the polysaccharide-protein type of antigen. Evidence for the existence of a conjugated protein common to the antigenic complexes present in other types of gram-negative bacteria is being sought and it is of interest in this connexion to record that 'Shiga' undegraded polysaccharide combines with the conjugated protein component of the 'O' antigen of *Bact. typhosum* to form a powerful antigenic complex that induces the formation of specific 'Shiga' agglutinins and precipitins. Thus, the capacity of a specific polysaccharide of one organism to form an antigenic complex when combined with the conjugated protein component derived from the antigen of another organism that belongs to an entirely different bacterial species, has been demonstrated.

An additional method for the isolation of the polysaccharide and protein components of the native antigenic complex is also described. Antigenic material dissolved in dilute NaOH is dissociated into its two main components. If the alkaline treatment is carried out quickly and at 0° an undegraded polysaccharide is recovered on the addition of alcohol whereas the protein component can be subsequently obtained from the alcoholic supernatant fluid by the addition of acetic acid. The protein recovered appears to be identical with the simple amphoteric substance obtained by phenol treatment of the whole antigenic complex.

The polysaccharide obtained by either phenol or alkali dissociation is found to yield viscous solutions in water and to contain 0·5–1·0 % of P in organic combination. The P is not removed by the action of bone phosphatase, prolonged dialysis or by precipitation of the material from solution in ice-cold 0·05*N* HCl by alcohol, from which it is inferred that the P is most probably an integral part of the polysaccharide molecule. Preparations of the degraded polysaccharide [Morgan, 1936] contain no appreciable quantity of P.

The viscosity values obtained for the undegraded polysaccharide exhibit a considerable variation according to the method of preparation used. It was at first thought that a determination of viscosity alone would furnish a reliable guide to the degree of degradation of the sample of polysaccharide, but it has been found that samples prepared by the phenol method occasionally have viscosity values only slightly greater than the degraded polysaccharide prepared by heating the antigenic complex in dilute acetic acid. It is to be noted, however, that phenol-treated preparations still contain 0·5–1·0 % P in organic combination and that in spite of the low viscosity, the material enters into union with the conjugated protein to yield an antigenic complex. A rapid test for ascertaining the potential capacity of polysaccharide preparations to form antigenic complexes is obviously of considerable practical value, and the most reliable guide so far found depends on the formation of soluble complexes with proteins at *p*H 4–5. On the addition of trichloroacetic acid to a mixture of conjugated protein and degraded polysaccharide a heavy precipitate of the protein immediately appears, whereas if the polysaccharide is in a form that will give rise to an antigen in combination with the conjugated protein, the solution remains clear, or, at most, develops a bluish opalescence.

It was earlier reported [Morgan & Partridge, 1940, 1; Partridge & Morgan, 1940] that the undegraded specific polysaccharide hapten of *Bact. dysenteriae* (Shiga) could be recombined with the non-antigenic conjugated protein ('polypeptide-like') component of the native antigen to form an antigenic complex that was able to induce the formation of specific 'Shiga' precipitins and agglutinins. Similarly, the conjugated protein component derived from the 'O' antigen of *Bact. typhosum* also combines with the 'Shiga' polysaccharide and forms an antigenic complex with 'Shiga' specificity. The simple protein derived

from the conjugated protein by the action of phenol, however, does not give rise to an antigenic complex in this manner and, in this respect, the simple amphoteric protein behaves like the proteins, stromatin, gelatin, human serum-γ-globulin, hen's egg vitellin and heat-denatured normal rabbit serum proteins. These observations suggest that a part of the conjugated protein molecule—most probably the prosthetic group—is necessary in order that the molecule should be capable of combining with the polysaccharide to form an antigen. The formation of a complex between undegraded specific polysaccharide and the different proteins examined certainly occurs as is shown by the behaviour of mixed solutions of the protein and polysaccharide on the addition of trichloroacetic acid. It appears unlikely that the stability of the protein under these conditions is due to the specific polysaccharide acting simply as a protective colloid, for gum acacia and other gums, which have low gold numbers [Schultz & Zsigmondy, 1903] and might be expected to function as powerful protective colloids, fail to prevent precipitation of the protein in the presence of trichloroacetic acid. In the light of these observations on the stability of proteins in the presence of an undegraded bacterial polysaccharide it is clear that the absence of a precipitate on the addition of the usual acid precipitants cannot be accepted as evidence that protein is not present in the material examined, and thus many reported observations on bacterial and tissue antigens stated to be 'protein-free' on the basis of negative tests given by trichloroacetic acid must be open to doubt. A more detailed study of these soluble and stable polysaccharide-protein complexes is being undertaken in collaboration with Dr R. A. Kekwick.

The possibility of evolving a differential extraction method whereby several organic solvents are utilized in turn for the solution and isolation of specific constituents of the bacterial cell, has already been discussed in an earlier paper [Morgan, 1937]. The results of some simple experiments along these lines are now described and it has been shown that after 6–7 % of the dry weight of the organisms employed has been removed by extraction with diethyleneglycol, it is possible by means of further extraction with strong solutions of phenol, to recover an additional 4–5 % of antigenic material together with about 10 % of a bacterial somatic protein. The antigenic material extracted from dry bacteria by 90 % phenol solution and recovered by precipitation with alcohol appears to be very similar in composition to the phospholipin-free primary extraction product obtained by means of diethyleneglycol. The free protein recovered from the bacilli, on the other hand, does not appear to be identical with the conjugated protein component of the antigenic complex. Preparations of protein which have been extracted by phenol from both 'rough' and 'smooth' cultures of *Bact. dysenteriae* (Shiga) contain 13·5–14·5 % N, are free from P and show a strongly negative rotation $[\alpha]_{5461} - 82 \pm 5°$. The protein isolated in each instance is electrophoretically homogeneous and represents at least 10 % of the dry weight of the bacterial cell.

Finally, it may be said that in common with most workers we have deemed a preparation derived from bacteria to be devoid of antigenicity when it failed to yield demonstrable homologous specific agglutinins and precipitins after it had been injected intravenously into each of a group of rabbits. Usually six doses of 0·05 mg. were given. This amount of the native antigen invariably elicits a powerful immune response with the resulting production of high titre agglutinating sera in all rabbits inoculated. For the purposes of direct comparison with other preparations from the same organism this method of measuring the antigenicity of a substance has yielded useful results, but it is necessary to emphasize that in the absence of adequate criteria of exactly what constitutes

an antigenic response it is advisable to keep in mind the results of other workers [Avery & Goebel, 1933; Chow, 1936; and Topley, 1937] who claim to have demonstrated the presence of protective immune-body in the serum of animals after immunization while being unable to detect the presence of the corresponding agglutinins or precipitins. In the absence of the latter immune-bodies the difficulty of deciding whether a preparation retains a part of the original antigenicity is still further increased when the material under test has been isolated from an organism such as Shiga's bacillus, for which the usual active and passive immunity tests that employ infection of the test animal with the living bacterium cannot readily be performed. Furthermore, it is only when adequate methods of assay exist that it is possible to detect certain changes in the qualitative nature of the immune-bodies formed. For example, Felix & Bhatnagar [1935] found that the Vi antibody elaborated in response to immunization with formolized Vi antigen is not identical with that resulting from immunization with native antigen as it occurs in the living, virulent *Bact. typhosum*. The quantitative production of Vi agglutinins appears to be normal when either type of vaccine is employed for immunization, but when sera of equal agglutinin contents are used, the phagocytosis-promoting activity and the protective power of the immune serum produced against the formolized Vi culture are much inferior to that induced after immunization with the living organism. Similar results were obtained by Henderson & Morgan [1938] who immunized rabbits with antigenic material isolated from *Bact. typhosum*. Once again, Vi agglutinins appeared in the immune serum but the protective power of the immune body as measured in passive protection tests was found to be much inferior to that given by an equal quantity of serum of equal Vi agglutinin titre prepared by immunization with living 'rough' Vi bacilli.

Summary

1. The antigenic complex of *Bact. dysenteriae* (Shiga) is soluble in concentrated (90 %) solutions of phenol. In phenol solution the complex is largely dissociated into its protein and polysaccharide components which can then be isolated in a free state by dialysis of the phenol solution.

2. The antigenic complex is partly dissociated in aqueous alkaline solution and the components can be separated by repeated fractionation of the antigenic complex from alkaline solution by alcohol.

3. The protein-free polysaccharide component prepared by either method fails to induce the formation of specific 'Shiga' agglutinins or precipitins in the rabbit.

4. Some evidence is given for the view that the acidic protein component previously described by the term 'polypeptide-like' is a conjugated protein. It is devoid of antigenicity as measured by the production of 'Shiga' immune-body, but can be recombined in aqueous solution with undegraded 'Shiga' specific polysaccharide to form a powerful antigen.

5. The protein isolated from the antigenic complex by phenol or alkali dissociation is amphoteric and has a higher nitrogen content and optical rotation than the conjugated protein. The simple amphoteric protein can also be isolated directly from the conjugated protein component of the antigenic complex. The simple protein does not form an antigenic complex when mixed with 'Shiga' polysaccharide in aqueous solution.

6. The extraction of a 'smooth' culture of *Bact. dysenteriae* (Shiga) with 90 % phenol yields the characteristic water-soluble antigenic complex and about an

equal amount of the non-specific, water-insoluble simple protein. Similar extraction of the corresponding 'rough' variant yields no antigenic material but a considerable quantity of the simple protein.

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