27. STUDIES IN IMMUNOCHEMISTRY

4. THE FRACTIONATION AND NATURE OF ANTIGENIC MATERIAL ISOLATED FROM *BACT. DYSENTERIAE* (SHIGA)[1]

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THE work of Boivin *et al.* [1933; 1934; 1935; 1937], Raistrick & Topley [1934], Topley *et al.* [1937], Mesrobeanu [1936], Morgan [1937], Henderson & Morgan [1938] and Miles & Pirie [1939, 1, 2, 3] has shown that the antigens of certain Gram-negative micro-organisms can be isolated in a relatively pure condition and apparently unchanged in their specific immunological properties. A method for the isolation of the antigenic complex from two such organisms—*Bact. dysenteriae* (Shiga) and *Bact. typhosum*—by extraction of the dry bacteria with anhydrous diethyleneglycol has already been described by Morgan [1937] and Henderson & Morgan [1938]. In the present communication the results of further work based on material isolated by the use of this method are given and evidence as to the chemical and physical nature of the dominant somatic antigen of the "smooth" strain of *Bact. dysenteriae* (Shiga) is discussed.

Before a more detailed investigation into the chemical nature of the antigenic material could be profitably undertaken it was considered advisable to demonstrate as conclusively as possible the homogeneous nature of the antigenic preparation selected for study and with this aim in view a number of experimental procedures were elaborated and applied. It would appear, however, to be an open question whether our present-day knowledge of the chemistry and physics of large molecular complexes will enable a decision on this point to be reached. In consequence it will be appreciated that in the absence of any recognized technique whereby naturally occurring colloidal complexes of similar nature may be separated from one another, some of the conclusions reached concerning the nature of the specific antigen may need subsequent modification. In the meantime, as further work on this subject is unavoidably postponed, the results of the investigation so far as completed are set out in this paper. A preliminary account has already appeared; Morgan & Partridge [1939, 2].

EXTRACTION AND PURIFICATION OF THE ANTIGENIC COMPLEX

The method used for the extraction of the antigenic complex has been described [Morgan, 1937]; additional details are now given. The preparation of bacteria of the "smooth" strain of *Bact. dysenteriae* (Shiga) was extracted 4 times with about 10 times its weight of anhydrous diethyleneglycol (B.P. 244–246°). Each extraction period lasted 4 days. The glycol suspension of the bacteria was mixed with 0·1 vol. of methyl alcohol, in order to reduce the density and

[1] For Part III see Morgan [1938, 1].

viscosity of the fluid medium and was centrifuged free from organisms in a Sharples supercentrifuge. A clear supernatant fluid which would readily pass through a British Berkefeld candle could usually be obtained after centrifuging for 1 hr. at about 35,000 r.p.m. After filtration the diethyleneglycol solution of the antigen was dialysed at 0° to eliminate the organic solvent and the antigenic material was obtained as a colloidal aqueous solution. The material was concentrated *in vacuo* at 10–15° until the solid content was between 1 and 2% whereupon the solution was centrifuged to remove any insoluble matter, cooled to 0° and treated with oxalic acid until no further insoluble material separated after standing at 0° for 24 hr. The deposit was removed by centrifuging and the excess oxalic acid was eliminated by dialysis against ice-cold distilled water. The antigen solution was treated at 0° with an equal volume of acetone which had been previously cooled to −10°; only a small quantity of material usually separated. The centrifuged solution was then treated with a volume of cold acetone equal to that of the aqueous antigen solution which immediately caused the precipitation of the main part of the antigenic material. The supernatant solution (66% acetone) was concentrated *in vacuo* and subsequently worked up for further antigen recovery when several batches of material became available. In order to eliminate the agar-like products that are present in the original antigen preparation at least two further acetone fractionations, between the levels 50 and 60%, were carried out on the redissolved 50–66% acetone precipitate. The material was then collected by centrifuging, resuspended in distilled water and dialysed free from acetone; it was ultimately obtained in the solid form by freezing the aqueous solution at −20° and allowing the frozen material to evaporate at room temperature *in vacuo* over H_2SO_4. Antigenic material prepared in this manner was used in all the experimental procedures that will now be described.

Before passing on, however, it is of interest to record the results obtained in diethyleneglycol extraction experiments in which several hundred g. of dry bacteria have been used. Earlier experiments [Morgan, 1937] have already shown that the material prepared as described above contains almost the whole of the specific antigenic complex present in the organism. It will be seen from the results summarized in Table 1 that the yield of antigenic material extracted

Table 1. *Antigenic material extractable with diethyleneglycol from the "smooth" form of* Bact. dysenteriae (*Shiga*)

Exp. no.	19	20	21	26
Total weight of dry bacteria (g.)	50	50	64	60
Extraction no. 1	1·29	1·59	2·00	2·10
,, 2	0·69	0·53	0·67	1·90
,, 3	0·22	0·30	0·65	0·58
,, 4	0·14	0·20	—	0·15
Total material extracted (g.)	2·34	2·62	3·32	4·73
,, ,, (% on dry bacteria)	4·7	5·2	5·2	7·8

by diethyleneglycol and purified by subsequent fractionation between the limits 50 and 60% acetone falls off rapidly with each successive treatment. Three successive treatments with diethyleneglycol, each of 4 days' duration, removed approximately 60, 25 and 12% respectively of the total antigen extractable under these conditions. The results also show that 5–7% by weight of the dried bacteria is recovered by this procedure and it would appear that this figure represents approximately the amount of antigen present in the dry organism.

Further extraction with diethyleneglycol, especially after prolonged contact lasting up to several weeks, yields some more material but the properties of the extracted substance show it to be quite distinct from the specific antigen of the "smooth" strain. This additional material, which evidently resides deep in the body of the organism, can only be reached after the surface antigen has been removed and is most probably, in part at least, an antigen characteristic of the "rough" strain. The yield of this material, even after prolonged extraction, amounts to little more than 1 % of the dry weight of the organisms extracted, but its presence in a diethyleneglycol extract suggested that a preparation of bacteria of the corresponding "rough" strain (K 624) should be subjected to a diethyleneglycol extraction process identical with that employed for the preparation of the specific "smooth" antigen. Accordingly, for control purposes, an extraction experiment was carried out with 45 g. of dry "rough" *Bact. dysenteriae* (Shiga) which is known to lack the specific antigen of the "smooth" strain. The material extractable from the "rough" strain is composed of "rough" antigen [Meyer, 1930] and certain non-specific material which is derived from the body substance of the organism. These substances are presumably also components of the "smooth" organism. Table 2 shows clearly that the amount

Table 2. *Material extractable with diethyleneglycol from "smooth" and "rough" variants of* Bact. dysenteriae *(Shiga). (Strain K 624)*

	"Smooth" g.	"Rough" g.
Weight of organisms	77	45
1st extract	2·7	0·09
2nd extract	1·1	Trace
3rd extract	0·25	Trace
4th extract	0·03	—
Total material extracted	4·08	0·09
Total material extracted (% of dry organisms)	5·3	0·2

of material extractable from the "rough" strain, after the usual fractionation of the product from aqueous solution by acetone, is quite small, being of the order of 0·2 % of the weight of the organisms extracted. If it is assumed that a similar amount of material, other than "smooth" antigen, is likewise dissolved from the organisms of the "smooth" strain and is subsequently present in the antigen preparation, then the extent of contamination of the "smooth" antigen with "rough" antigen and with non-specific somatic material amounts at most to 3 or 4 %.

Fractionation with acetone. A preparation of antigen from the "smooth" strain obtained by diethyleneglycol extraction of the dry bacteria and fractionated twice with acetone between the levels 50 and 66 % by volume was repeatedly fractionated from 1 % aqueous solution with cold acetone in order to ascertain whether such treatment enabled the material to be separated into components that were chemically or immunologically distinct.

The antigenic material was suspended in distilled water to yield a 1 % colloidal solution. The opalescent solution was cooled to 0° and acetone at −10° was slowly added with constant shaking. The addition of an equal volume of acetone produced no visible precipitate but centrifuging caused about 5 or 10 % of substance to separate. When the acetone concentration reached 60 % by volume a large precipitate of the antigenic material separated and was collected by centrifuging. The supernatant fluid was made up to 66 % with acetone, a further fraction was removed and the acetone solution was concentrated under

reduced pressure at 20°, dialysed and dried. All fractions precipitated with acetone were resuspended in water and were dialysed at 0° to remove acetone. The fractions were then obtained in the solid form by rapid evaporation *in vacuo* of the frozen solutions. The optical rotations were measured in neutral formamide. The material precipitated by 50 % acetone, however, frequently gave an opalescent solution in formamide and for this reason the specific rotation could only be approximately determined.

The material thrown out of solution between the levels 50 and 60 % acetone was usually found to make up 70–80 % of the antigenic substance employed and showed a slight opalescence in formamide solution; $[\alpha]_{5461} + 55 \pm 5°$ (c, 0·5). A small fraction, $[\alpha]_{5461} + 60 \pm 5°$, was precipitated between 60 and 66 % acetone and not more than 5 % of the original material was found to be soluble in the 75 % acetone supernatant fluid. The main fraction was again fractionated from 1 % aqueous solution by acetone. There was usually no precipitate at 50 % acetone but a large fraction was thrown out of solution when the acetone concentration was increased from 50 to 55 %. This fraction, in 1 % aqueous solution, was treated a third time with acetone but no material separated which showed a rotation outside the limits $[\alpha]_{5461} + 56 \pm 5°$. The antigenic material at this stage yields an almost clear, colourless solution in formamide. It was apparent that no further separation could be achieved by means of this technique and in one experiment, that involved four separate fractionation processes, 7 g. of the primary extraction product gave rise to 5·4 g. of antigenic material, $[\alpha]_{5461} + 56 \pm 3°$, which precipitated between 50 and 55 % of acetone. Other fractions obtained during the process showed extreme rotations which varied from +45 to +62° but subsequent treatment of these again yielded a main fraction which showed $[\alpha]_{5461} + 56 \pm 5°$ (c, 0·5 in formamide). A small amount of the specific polysaccharide component was found in the final acetone supernatant fluids.

Fractionation with alcohol. A similar series of fractionations was made in which absolute alcohol replaced acetone but no definite indication could be obtained that the antigenic material was composed of a simple mixture of different substances.

Fractionation with ammonium sulphate. Attempts were made to fractionate a specimen of the antigenic material by means of ammonium sulphate after the complex had been repeatedly precipitated from aqueous solution by acetone. The material (500 mg. in 50 ml. water) was almost completely thrown out of solution between the ammonium sulphate levels 21–23 % by volume. The supernatant fluid yielded only 38 mg. of antigenic material after it had been dialysed free from ammonium sulphate and evaporated to dryness. It would appear, therefore, that the antigenic material cannot be separated into several fractions that show different solubilities in ammonium sulphate solution.

Extraction with organic solvents. In view of the earlier work [Mesrobeanu & Calab, 1936; Morgan, 1937], which showed that a fatty substance could be obtained from the antigenic complex by acid hydrolysis, it seemed desirable at this juncture to prove that the fatty substance is not simply a loosely bound contaminant but is in fact a definite part of the antigenic complex. A preparation of the primary extraction product that weighed 6 g. was therefore extracted successively with (*a*) five portions each of 200 ml. of freshly distilled ether, (*b*) five portions each of 200 ml. of a 1 : 3 alcohol-ether mixture, (*c*) two 200 ml. portions of redistilled chloroform and finally a colloidal aqueous solution of the material was thoroughly extracted with freshly distilled ether. As a result of these extractions only 0·8 % of the total weight of the antigenic material

was found to be soluble. It would appear, therefore, that the fatty material which can be isolated in a yield of 9–12 % of the substance after acetic acid hydrolysis of the complex and also, as will be described later, by the action of formamide, is in fact a component of the antigen and is not merely adventitious material.

Antigenicity tests. Numerous antigenicity tests in rabbits were made with material obtained by acetone or alcohol fractionation and possessing a specific rotation in anhydrous formamide of $+56° \pm 5°$. In every preparation examined an immunizing course of three doses each of 0·01 mg. of the fraction under test gave rise to an immune-serum showing agglutination to high titre whereas precipitation, haemolytic and complement fixation tests, where these were made, indicated that the immune-serum produced was qualitatively identical with the antibacterial immune-body induced by a killed culture of a "smooth" strain of *Bact. dysenteriae* (Shiga). A more detailed account of the immunological aspects of this work will be given elsewhere.

Antigenic material that has been repeatedly precipitated from 1 % aqueous solution by acetone between the levels 50 and 60 % and which is called "the primary extraction product" appears therefore to be largely homogeneous in nature and free from non-specific substances. It cannot be stated, however, that this product is identical in its chemical and physical properties with the antigenic complex as it exists in the intact organism. Further observations on this point will be discussed later in the paper.

The properties of the primary extraction product

The substance in a concentration of 1 % gives rise to a heavy milky opalescent aqueous solution but dissolves readily in anhydrous formamide, formic acid (99–100 %) and concentrated phenol solution to give clear solutions. The material is insoluble in glacial acetic acid and dioxan. The opalescence in 6·6 M urea solution is considerably less than that in water; this is presumably due to disaggregation of the complex into smaller units. The antigenic material after disaggregation in strong solutions of urea or formic acid can be obtained largely in a reaggregated form by removing the urea or formic acid by dialysis and recovering the antigen in the dry condition; re-solution of the material in water again gives rise to a strongly opalescent solution. A determination of the sedimentation factors under appropriate conditions with a view to ascertaining the particle size of the primary extraction product both before and after treatment with different solvents is being undertaken in collaboration with Dr A. S. McFarlane and the relation of changes in the size of the antigen particle to the biological activity of the product is also under investigation.

A 1 % solution of the primary extraction product after heating in 1 % acetic acid at 100° for 30 min. commences to flocculate and the flocculation process is complete in about 1 hr. The same antigenic preparation, however, when heated in neutral aqueous solution under the same conditions, shows no evidence of flocculation up to a period of at least 3 hr. After this time the colloidal properties of the complex are destroyed and precipitation of the polypeptide and phospholipin components occur. The loss of antigenicity during heating appears to take place before flocculation and in one experiment antigenic material that had been heated at pH 7 for 1 hr. was found to induce in rabbits no demonstrable precipitins for the specific polysaccharide after the usual three intravenous doses each of 0·05 mg. had been given. It is of interest to record that the phospholipin component of the antigenic complex is not removed from the complex by extraction with ether after the material has been heated at 100° for 1 hr.,

that is, after the material has lost its antigenic properties but before flocculation has occurred.

Preparations of antigenic material that have been fractionated from 1% aqueous solution 2 or 3 times by means of acetone according to the technique described have always been found to be free from agar or kanten according to the three colour tests elaborated by Pirie [1936]. The primary extraction product (10 mg.) yields a reddish-purple colour whereas the same quantity of the phospholipin-free complex (to be mentioned later) gives a purple colour only with the carbazole reagent. Under the same conditions agar gave rise to a very dark blue coloration in a much shorter time. It should be mentioned in connexion with the carbazole test for agar that glucosamine and chondrosamine also give greenish-blue colorations which, however, are much less intense than the purer green colour given by an equal weight of agar or its derivative. The diphenylamine reagent gives rise to no appreciable colour with the antigenic material (10 mg.) in a time of heating which produces a strong green colour with about 1 mg. of agar. It has already been stated [Morgan, 1937] that anti-agar horse immune-serum gives no precipitin reaction with the antigenic material. The results of these experiments indicate that the antigen preparations can contain at the most only an insignificant quantity of agar or kanten.

The general chemical properties of the primary extraction product have already been described [Morgan, 1937] together with a preliminary account of the nature of the acid hydrolysis products. This part of the work will now be described in greater detail.

Hydrolysis of the primary extraction product

The antigenic material (3·0 g.) was shaken with water (150 ml.) until the material was redissolved. The material, which dissolved completely, could not be thrown out of solution by centrifuging at normal speeds (2000–3000 r.p.m.). Acetic acid was added to make a 0·1 N solution and the colloidal solution was heated at 95–98° on a boiling water bath for 4 hr. After 30 min. some insoluble material had separated and the opalescent solution subsequently became clear after a further 2 hr. heating. The solution was cooled and kept at 0° overnight after which time a thin film of white semi-solid fatty material had separated as a surface layer on the colourless aqueous solution and a heavy white precipitate was present in the bottom of the flask.

The hydrolysis products were repeatedly extracted with freshly distilled ether until no further material could be removed. The ether extract was dried over anhydrous Na_2SO_4, filtered and immediately evaporated to dryness *in vacuo*. The ether-soluble substance (Fraction I, 0·315 g.) represented 10·5% of the total antigen. The material was redissolved in pentane and filtered before the final process of evaporation and weighing was carried out.

The aqueous solution, after warming to 40° under reduced pressure to remove ether, was cooled to 0° and the precipitate collected by centrifuging, washed twice with 5 ml. of ice-cold distilled water and dried *in vacuo* over $CaCl_2$. The material (Fraction II, 0·65 g.) was a buff-coloured powder and represented 21·7% of the total antigen. In other experiments somewhat lower figures were obtained for this fraction.

The aqueous solution and washings were evaporated to about 25 ml., treated with 400 ml. of absolute alcohol and kept at 0° for about 18 hr. The precipitated polysaccharide was collected by centrifuging, washed several times with 95%

alcohol, then with absolute alcohol and finally dried *in vacuo*. The material was dissolved in 10 parts of water, a small insoluble residue was removed and the polysaccharide was again precipitated by the addition of 10 vol. of absolute alcohol, washed with 95 % and absolute alcohol and dried *in vacuo*. The white amorphous polysaccharide material (Fraction III, 1·52 g.) represented 50·5 % of the total antigenic material hydrolysed.

The alcoholic supernatant solution together with the washings were evaporated to dryness *in vacuo*; the material (Fraction IV) weighed 0·46 g. and represented about 15 % of the antigenic complex. The material gives a test for arginine but does not appear to contain any appreciable amount of Fraction II (polypeptide-like component). The material reduces Fehling's solution only after acid hydrolysis.

The results obtained from many similar hydrolysis experiments, but using smaller quantities of antigen, suggest that the following values represent the ranges of variation to be expected for each component. Fraction I, ether-soluble material, 9–12 %; Fraction II, insoluble in dilute acid, 17–20 %; Fraction III, 50–55 %.

The difficulties of separating such complex substances quantitatively and in a pure condition are well known and it is not claimed that the figures given are as accurate as those obtained in estimations involving an accepted quantitative procedure. The method of isolation, however, once chosen, was adhered to throughout the work and the purity of the components finally isolated was checked by N or P determinations. Losses due to unavoidable decomposition and partial solubility must be expected and the figures given almost certainly represent minimum values. The values given for the amount of polysaccharide are known to be 10–20 % too low. This correction is based on the results of experiments made to determine the percentage recovery of small quantities of the pure polysaccharide when it is isolated under conditions similar to those existing after the acid hydrolysis of the antigenic complex.

Examination of the ether-soluble component (Fraction I) of the antigenic complex

Saponification. A portion (0·178 g.) of the ether-soluble material (Fraction I) was dissolved in pentane (8–10 ml.) and shaken thoroughly with 5 ml. of 5 % Na_2CO_3 and subsequently with three 5 ml. amounts of distilled water. The combined aqueous extracts were acidified with acetic acid and extracted with freshly distilled ether, the ether was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. About 20 mg. of a white crystalline organic substance were obtained, representing 11 % of Fraction I; although this fraction has not been examined in detail the evidence so far available indicates that it most probably consists of mixed fatty acids.

The pentane solution after treatment with aqueous Na_2CO_3 and extraction with water as described above was dried, filtered and evaporated to dryness. A white waxy solid (137 mg.) was obtained; $[\alpha]_{5461} + 12° \pm 2°$ (*c*, 1 in pentane). Total P, 3·9 %; inorganic P, nil. N, 1·8 % (Kjeldahl). Average analytical figures given in the literature for the phosphatide, kephalin; P, 3·7–4·0 %; N, 1·7–1·8 %. The P : N ratio is thus in both cases almost exactly unity, which indicates that the fatty material under examination probably belongs to the monoaminophospholipin group of substances. The phosphatide is only sparingly soluble in absolute alcohol and in this property it resembles kephalin rather than lecithin.

The phosphatide material was dissolved in 3 ml. of methyl alcohol which contained 140 mg. of KOH and was saponified by heating on a boiling water bath for 1 hr. under reflux. The cooled solution was diluted with 9 ml. of distilled water and was thoroughly extracted 4 times with 25 ml. amounts of ether; the ether was then extracted 3 times with 3 ml. quantities of 25 % methyl alcohol, once with 1 ml. of dilute NaOH and again with 3 portions of 25 % methyl alcohol.

The ether, after extraction, contained the unsaponifiable matter present in the original fatty material; this amounted to only 5 mg. and represented approximately 3 % of the original ether-soluble fraction.

The main 25 % methyl alcoholic solution and the combined 25 % methyl alcoholic extracts were freed from methyl alcohol by heating *in vacuo*, the aqueous solution was acidified with dilute H_2SO_4 until just blue to Congo red (*p*H 4) and it was thoroughly extracted with pentane to remove free fatty acids. The pentane solution was dried over anhydrous Na_2SO_4, evaporated to dryness and sealed in an ampoule *in vacuo*. The white, semi-solid and partly crystalline material so obtained consisted of the mixed fatty acids derived from the phosphatide, weighed 89 mg. and represented 65 % of the total pentane-soluble hydrolysis products.

Separation of the fatty acids. A separation of the mixed fatty acids was carried out by the method of Twitchell [1921] in which the saturated and unsaturated acids are isolated according to the solubility of their lead salts in 95 % alcohol. The total material (89 mg.) was dissolved 95 % alcohol (1 ml.), heated to boiling point and treated with 1 ml. of a boiling 95 % alcoholic solution of lead acetate. The mixed solutions were allowed to cool slowly and to remain at room temperature for at least 2 hr.

The isolation of palmitic acid. The crystalline lead salt that separated was filtered off, washed with 95 % alcohol and absolute alcohol and finally dried *in vacuo*. The material, which weighed 51 mg. and melted sharply at 103–104° (corr.), was suspended in water, treated with dilute HCl to remove Pb and thoroughly extracted with ether. The ether extract was then washed with water until the washings were neutral, dried over anhydrous Na_2SO_4 and evaporated to dryness whereby 25 mg. of a fatty acid, M.P. 49–54°, were obtained. Recrystallization of the acid from acetone at $-80°$ finally gave a substance which melted at 58–60° (corr.) and showed no depression of M.P. when mixed with pure palmitic acid (M.P. 59–61°) whereas a specimen mixed with stearic acid melted several degrees lower. A portion of the acid was dissolved in 5 ml. of freshly distilled ether and treated with an ethereal solution of diazomethane until a permanent yellow colour persisted. After 10 min. the solution was evaporated to dryness *in vacuo* and the crystalline residue was recrystallized from ether-pentane. The methyl ester crystallized in needles, M.P. 26–29°, and a mixed M.P. of the ester with the corresponding ester of palmitic acid (M.P. 28–31°) melted at nearly the same temperature (27–29°). Palmitic acid is therefore considered to be a component of the phospholipin present in the antigenic complex.

Isolation of oleic acid. The Pb salt soluble in cold 95 % alcohol, together with the alcoholic washings obtained during the isolation of the Pb salt of palmitic acid, were immediately decomposed by treatment with dilute HCl and the free fatty acid recovered by extraction with ether. The ethereal solution after being washed free from mineral acid with water was dried over anhydrous Na_2SO_4 and evaporated to dryness *in vacuo*; the material was stored in a sealed ampoule containing CO_2. The fatty acid (58 mg.) was obtained as a pale yellow oil.

The material was dissolved in absolute ether (2–3 ml.) and after cooling to $-10°$ was treated with an excess of bromine which was also dissolved in dry ether. The mixture was shaken and kept at $-10°$ for 2 hr. but the separation of an insoluble hexabromide [Hazura, 1887] did not occur. A control experiment in which 50 mg. of linolenic acid were similarly treated gave a good yield of the insoluble hexabromide. The ether was removed *in vacuo* and was replaced by pentane but the material was again found to be completely soluble. A sample of linoleic acid when treated in this manner gave a good yield of the pentane insoluble tetrabromide. The results of these experiments indicated that linoleic acid and linolenic acid were not present in appreciable quantities in the fatty material under examination. The liquid dibromide of oleic acid is readily soluble in ether or pentane and cannot be isolated by this method.

The pentane solution which presumably contained a soluble bromo derivative was freed from bromine by heating under reflux for 1 hr. with 100 mg. of zinc powder and 3 drops of glacial acetic acid. The solution was cooled and, together with the insoluble residue in the flask, was thoroughly extracted with dilute HCl in presence of ether. The ethereal extracts were dried over Na_2SO_4 and evaporated to dryness *in vacuo* when about 37 mg. of a pale yellow oil were obtained. The oil was dissolved in the minimum amount of ether in an atmosphere of CO_2 and was sublimed at 0·005–0·01 mm. Three fractions were collected: (1) bath temperature 30–70°, 3 mg.; (2) bath temperature 70–130°, 28 mg.; (3) bath temperature 130–160°, about 5 mg. There was only a trace of residue after sublimation. The middle fraction was dissolved in light petroleum (B.P. 60–80°) and heated under reflux with thionyl chloride until evolution of HCl had ceased; during this process moisture was rigorously excluded. The petroleum solution of the acid chloride of the unsaturated fatty acid was evaporated to dryness *in vacuo*, the petroleum replaced by 10–15 ml. of freshly distilled ether and an ethereal solution of an excess of *p*-phenylaniline was added. After keeping the solution at room temperature for 10 min. the excess of reagent was removed by treatment with dilute HCl. The ethereal solution of the derivative was then washed with dilute NaOH, finally with water and, after drying over anhydrous Na_2SO_4, the *p*-phenylanilide of the fatty acid was obtained crystalline by removing the solvent *in vacuo*. After one recrystallization from methyl alcohol at low temperature, the derivative melted at 120–121°. Yield 30 mg. The *p*-phenylanilide of oleic acid melts at 120–121° [Kimura & Nihayashi, 1935] and a mixed M.P. of this substance with the *p*-phenylanilide of the unknown fatty acid melted at the same temperature. The unsaturated fatty acid present in the phospholipin complex appears to be oleic acid.

The presence of other organic acids. The aqueous solution after extraction with pentane in order to remove the fatty acids liberated during the saponification of the phosphatide was extracted with ether to isolate lower aliphatic acids or dicarboxylic acids such as succinic acid, should these be present. The ether extract, however, yielded only a trace of material on evaporation to dryness from which it appears that simple aliphatic and other acids are not present in the hydrolysis products.

The isolation of glycerophosphoric acid. Examination of the acidified aqueous solution derived from the alkaline hydrolysis products of the phosphatide, after it had been thoroughly extracted with pentane and ether to remove the saturated and unsaturated fatty acids, showed that about 2·5 mg. of organically bound phosphate were present. Inorganic phosphate was absent. A small portion of the solution was concentrated to dryness and heated in a tube with a small piece of filter paper previously moistened with a freshly prepared 2 % alcoholic solution

of piperidine which contained a drop of a solution of sodium nitroprusside. The filter paper developed a bright blue colour which on subsequent treatment with dilute alkali immediately changed to a reddish-purple. According to Feigl & Frehden [1937] this test is specific for glycerol. The main part of the solution was therefore exactly neutralized by the cautious addition of KOH; it was evaporated to about 0·5 ml., 20 vol. of absolute alcohol were added and the solution was kept at 0° for some hours in order to throw out of solution as much K_2SO_4 as possible. The solution was filtered from K_2SO_4, concentrated to a small volume and the resulting syrup taken up in methyl alcohol and treated with methyl alcoholic barium acetate and a drop of baryta solution until precipitation of the Ba salt was complete. The solution was kept at 0° overnight, after which time the supernatant fluid was found to be almost free from P. The Ba salt was filtered off, washed with a small volume of 90 % methyl alcohol to remove barium acetate and with absolute alcohol and dried *in vacuo*. The Ba salt (25 mg.) was dissolved in the minimum quantity of boiling distilled water and filtered from traces of insoluble matter. The colourless filtrate was allowed to cool slowly and was kept at 0° overnight when a Ba salt separated from solution. The substance was filtered off, washed with 90 % alcohol and finally with absolute alcohol and dried *in vacuo*. A solution of the Ba salt, after heating at 100° for 20 min. with $2N$ HCl failed to reduce Fehling's solution, from which it was inferred that hexose- or pentose-phosphoric acids were absent. Analysis of the Ba salt gave: Total P, 9·1; inorganic P, nil. Ba, 39·6 %. Ratio, Ba : P, 1 : 0·229. Ba glycerophosphate $C_3H_7O_6PBa, H_2O$ requires P, 9·5. Ba, 42·1 %. Ratio, Ba : P, 1 : 0·225. The analytical figures indicate that the Ba salt was not quite pure but this cannot be considered surprising owing to the difficulty of effecting a thorough purification when only a few mg. of material are available.

In the presence of $BaNO_3$ the Ba glycerophosphate which had been isolated failed to form a complex salt $2C_3H_7O_6PBa\text{-}Ba(NO_3)_2$ as described by Karrer & Salomon [1926] for the isolation and identification of Ba-β-glycerophosphate. A control experiment, in which only 5 mg. of an authentic specimen of the β-salt were employed, gave rise to a crystalline mass of the $BaNO_3$ complex within a few minutes of mixing the two solutions. On the other hand a similar experiment with twice this quantity of Ba-α-glycerophosphate failed to deposit an insoluble complex when treated with $BaNO_3$ solution. These experiments indicate that the glycerophosphoric acid present in the phospholipin fraction is essentially in the α-form.

The examination of the acid insoluble component (Fraction II) of the antigenic complex

Properties

Earlier work [Morgan, 1937; 1938, 2] has already shown that the material which remains insoluble in dilute acetic acid after the products of acid hydrolysis have been thoroughly extracted with ether or pentane is most probably polypeptide in nature.

The material is an acidic amorphous substance which yields a clear viscous solution in dilute alkali and in Na_2HPO_4 buffer (pH 8·4). The substance is almost completely insoluble in dilute acid, but is soluble in 30 % H_2SO_4. The analytical figures for different preparations vary slightly but representative values are: C, 44; H, 6·7; N, 11·5 % $[\alpha]_{5461} -48°$ (c, 0·5 in $0{\cdot}2N$ NaOH). P accounts for less than 0·2 % of a carefully purified specimen from which it appears that P is not a normal constituent of the polypeptide component.

The polypeptide gives a positive biuret test and a strong test for arginine (Sakaguchi reaction). After acid hydrolysis, however, the biuret test becomes negative and the hydrolysis products give an intense yellowish-red coloration with diazobenzenesulphonic acid in alkaline solution, a positive α-nitroso-β-naphthol test for tyrosine and a strong ninhydrin reaction. One preparation which had been purified from small amounts of the other components of the antigenic complex induced in rabbits traces only of precipitins for the polypeptide itself, whereas no immune-body against the specific polysaccharide, the whole antigenic complex or *Bact. dysenteriae* (Shiga) could be demonstrated by precipitation or agglutination tests respectively. The material dissolved in phosphate buffer at *p*H 8·0 is not coagulated on boiling. An alkaline solution of the polypeptide dialyses through a cellophane membrane only very slowly. In one experiment, in which the polypeptide component was dissolved in phosphate buffer at *p*H 8·0 and dialysed against the same buffer at 0°, 25 % of the total polypeptide diffused during 7 days. After 5 days more 33 % of the polypeptide had diffused and at the end of 18 days 42 % had passed through the membrane. The experiment was then discontinued. The diffusate and the substance remaining in the bag gave strong colour reactions for arginine.

The acid hydrolysis of the polypeptide. Hydrolysis with $2N$ HCl at 100° is difficult owing to the low solubility of the polypeptide in dilute mineral acid. Furthermore, the prolonged hydrolysis that becomes necessary under these conditions induces considerable discoloration. The hydrolysis is therefore best carried out in $10N$ formic acid which contains 3·6 % HCl. The acid mixture readily dissolves the polypeptide and the solution develops only a very slight yellow colour during the hydrolysis. The *p*H of the acid mixture is approximately equivalent to that of N HCl. With this acid mixture it is advisable to carry out the heating in small sealed tubes completely immersed in a boiling water bath. Under these conditions the hydrolysis, as measured by increase in free amino-groups (Van Slyke), is complete in 2 hr. and the amino-N increases during the hydrolysis from less than 10 % to about 50 % of the total N. In all determinations the formic-HCl mixture was exactly neutralized before the Van Slyke determination was made and numerous blank experiments were included. According to the colorimetric method of Folin & Ciocalteu [1927] for the estimation of tyrosine, the polypeptide contains about 8·0 % of this amino-acid and at least 5·5 % of arginine, as determined by the quantitative procedure of Weber [1930], is also present in the hydrolysis products.

Tryptic digestion of the polypeptide. The polypeptide is readily attacked by trypsin in alkaline solution (*p*H 8·5) and subsequent acidification of the solution no longer precipitates unchanged amorphous polypeptide. The polypeptide (N, 11·6 %) was dissolved in Na_2HPO_4 buffer (*p*H 8·4) to yield a solution that contained 13 mg. per ml. and 2 mg. of a commercial trypsin preparation were added to each ml. After keeping at 37° for 12 hr. a further equal quantity of trypsin was added and the incubation continued for 12 hr. more. At the end of this time the polypeptide was not thrown out of solution when the mixture was acidified whereas from the control solutions the polypeptide was precipitated unchanged. A determination of the free NH_2 groups (Van Slyke) showed that after tryptic hydrolysis 46 % of the total N was in this form.

A more detailed study of the polypeptide-like component (Fraction II) is being undertaken.

The polysaccharide component (Fraction III) of the antigenic complex

This fraction has already been described in some detail [Morgan, 1936; 1938, 1] and has been shown to consist, in part at least, of *d*-galactose and *l*-rhamnose. The presence of an *N*-acetylhexosamine was also detected and according to a colorimetric method [Morgan & Elson, 1934] was estimated to represent between 20 and 25% of the polysaccharide complex. Several attempts to isolate and identify the hexosamine from the acid hydrolyses products of the polysaccharide failed, but since that time a method has been elaborated [Jolles & Morgan, unpublished results] for the isolation of small quantities of glucosamine and chondrosamine and it is now believed that the hexosamine is glucosamine. The polysaccharide that is isolated after acetic acid hydrolysis of the antigenic complex contains about 0·5% of P in organic combination. Full details of this part of the work will be given in a later paper.

Degradation of the antigenic complex

It would appear from the results of the experiments so far described that the antigenic material, that has been termed the primary extraction product, is built up from not less than three components: (1) the specific polysaccharide, (2) the polypeptide-like body and (3) a phospholipin.

In an attempt to obtain evidence which would indicate whether all of these components are necessary for the manifestation of antigenicity, methods have been employed by means of which either the phospholipin or the polypeptide can be eliminated from the antigenic complex as desired. As a result it has been possible to degrade the antigenic material in a stepwise manner and investigate the chemical and immunological properties of the resulting substances [Morgan & Partridge, 1939, 2]. Thus, by the action of trypsin on the antigenic material a product can be obtained which is almost free from polypeptide. Formamide treatment of the resulting polysaccharide-phospholipin complex eliminates the latter component and yields the specific polysaccharide. On the other hand, solution of the antigen in pure formamide with subsequent fractionation by means of alcohol eliminates the phospholipin and a complex is obtained which consists of polysaccharide and polypeptide [Morgan & Partridge, 1939, 1].

The dissociating action of formamide

Partial dissociation. A typical procedure adopted for the removal of phospholipin from the natural antigen was as follows. The antigenic material (2 g.) was dissolved in 120 ml. of anhydrous, neutral formamide (M.P. 1–2°)[1] and kept at room temperature for 1 hr. The formamide solution, which was somewhat opalescent, was centrifuged to remove traces of insoluble material and the supernatant fluid was treated with 2 vol. of ice-cold absolute alcohol. A small precipitate formed and was removed and the solution was treated with a further volume (120 ml.) of alcohol. This concentration of alcohol (75% by volume) precipitated from solution the greater part of the antigenic complex which was immediately collected by centrifuging, resuspended in water, dialysed and evaporated to dryness *in vacuo* below 0°. The 75% alcoholic supernatant fluid was found to contain practically the whole of the phospholipin component together with small amounts of phospholipin-free antigen and polysaccharide. Experience has shown that it is advisable to repeat the fractionation from

[1] Supplied by Dr Fränkel, Treforest Estate, near Cardiff.

formamide solution with the material that has been precipitated between 66 and 75 % alcohol concentration, as described above, in order to eliminate completely the last traces of the phospholipin.

An attempt was made to fractionate a preparation of phospholipin-free material. A 1 % aqueous colloidal solution of the material (50 ml.) was cooled to 0° and was treated with acetone. At the 60 % level a precipitate formed and was removed. Acetone was added until the solution contained 70 % when a further fraction was collected. The two main fractions were again fractionated from 1 % aqueous solution by acetone at 60 and 70 % levels and the first and last fractions were examined. The material (60 mg.) precipitated with 60 % acetone was found to yield 20 % of the polypeptide component and 57 % of polysaccharide after acid hydrolysis whereas the most soluble material (280 mg.) thrown out at 70 % acetone level contained 19 % polypeptide and 58 % polysaccharide. The original phospholipin-free material used in this experiment yielded, after acid hydrolysis, 20 % polypeptide and 61 % of polysaccharide. It would appear, therefore, that the original complex contained a slight excess of uncombined polysaccharide but was otherwise fairly homogeneous in character. A phospholipin-free complex of this nature, however, will only be obtained if the so-called natural antigen is exposed to cold, neutral formamide for a short time. The prolonged action of a large excess of neutral or slightly acid formamide will give rise to a main fraction that is not only free from phospholipin but is also seriously diminished in its content of polysaccharide. On the other hand treatment of the original antigenic complex with insufficient neutral formamide will restrict the dissociation of the components and give rise to a substance that will still contain a few per cent. of phospholipin. The more extensive dissociation of the antigenic material will be discussed again later.

The properties of the phospholipin-free complex. The properties of the phospholipin-free antigen are as follows. The specific rotation is somewhat higher than that shown by the so-called primary extraction product and is $+65° \pm 5°$ (c, 0·5 in formamide). Analytical figures for a typical specimen are: C, 42·3; H, 6·8; N, 4·4 (Dumas); ash 3·5 %. In such a preparation the acid-insoluble component (Fraction II) that can be isolated after acid hydrolysis amounts to approximately 22 % and the material gives strong biuret, α-nitroso-β-naphthol and Sakaguchi reactions. The phospholipin-free antigenic material gives an opalescent solution in water, but differs from the primary extraction product inasmuch as the addition of sufficient dilute alkali to raise the pH to 8 causes the solution to become quite clear whereas a solution of the original antigen remains distinctly opalescent after this treatment, although a considerable diminution in turbidity occurs. The addition of dilute acid to a solution of either preparation to lower the acidity to pH 1 causes only a slight decrease in opalescence. The phospholipin-free material is strongly antigenic and 3 doses, each of 0·01 mg. given intravenously to rabbits, induced the formation of potent immune-sera in each of the 6 animals immunized. In some sera an agglutination titre of 1 : 1280 was recorded and some of the immune-sera showed specific *Bact. dysenteriae* (Shiga) haemolytic antibody at a dilution of 1 : 2000. All the immune-sera gave immediate and heavy precipitates on addition of the homologous antigen, the primary extraction product (original antigenic material) or the specific polysaccharide. Although the phospholipin component seems, on the basis of these immunization experiments, to be unnecessary for the manifestation of antigenicity it is impossible to be certain that it has no immunological function. For example, it is conceivable that the natural antigen with its associated phospholipin component is a more potent antigen weight for

weight than the simpler polysaccharide-polypeptide complex. Unless, however, the difference in antigenic capacity of these two complexes is large there is no simple method available by means of which it is possible to obtain evidence in support of this suggestion.

A 1 % aqueous solution of the phospholipin-free antigen was centrifuged for 1 hr. at 14,000 r.p.m. in an Ecco-Blitz centrifuge in order to determine whether the material thrown out of solution or the material remaining in the supernatant fluid was antigenic. The greater part of the material was thrown down as a whitish gel whereas the supernatant fluid became quite clear and colourless. The precipitate was made up to the original volume in saline and tested, together with the supernatant fluid, for antigenicity. An excellent response was given by two rabbits that each received 3 doses of 0·05 mg. of the centrifuged deposit whereas no appreciable antibody production could be demonstrated in the sera of two other animals which received material that remained in the supernatant fluid. It should be mentioned, however, that although the supernatant fluid gave a positive Molisch reaction the amount of material contained therein represented only a few per cent. of the total antigenic material used in the experiment.

Complete dissociation. Repeated formamide treatment of the natural antigen, especially when the formamide contains 2–3 % of formic acid, carries the dissociation of the antigenic complex beyond the simple removal of the phospholipin. After the first formamide treatment the main part of the phospholipin-free material is precipitated from formamide solution by alcohol within narrower limits (66–75 % by volume) while the polypeptide component present in the precipitated material increases in amount with each formamide treatment. After two or three treatments with acid formamide the bulk of the material is precipitated from a 2–3 % solution of the preparation in formamide by 55–60 % of alcohol and this material may contain as much as 50 % of the polypeptide component (N, 11·6 %). Material of this nature possesses a much lower specific rotation ($[\alpha]_{5461}+30^\circ$ for one preparation) than the original phospholipin-free substance and represents a partially dissociated polysaccharide-polypeptide complex that has already lost a considerable part of its polysaccharide component. Further formamide treatment would probably eliminate the polysaccharide completely but the dissociation has not been continued to this point. The polysaccharide component liberated remains in the 75 % alcoholic formamide supernatant fluid and can be recovered by dialysis to remove formamide, concentration to a small volume and fractionation from aqueous solution by alcohol. Three doses, each of 0·01 mg. of the polysaccharide prepared in this manner, fail to produce in the rabbit demonstrable "Shiga" agglutinins or haemolysins. Two examples of the products arising from the partial dissociation of the phospholipin-free antigen preparation by means of neutral and acid formamide respectively are given in Table 3. The products A and C were precipitated by 66 % alcohol from neutral and acid formamide solutions of the antigenic material. The substances B and D remained in the alcoholic formamide. In this particular experiment, after equilibrium conditions had been established in the formamide solution, 66 % alcohol precipitated a product A which contained 28 % of polypeptide and at least 56 % of polysaccharide component whereas the more soluble material, B, contained 10 % of polypeptide and a much higher proportion of the polysaccharide component. On the other hand the nature of the products arising from the dissociating action of the acid formamide indicated that a much increased dissociation of the complex had occurred, and in this experiment the material precipitated by 66 % alcohol, fraction C,

Table 3. *Products of the partial dissociation (2nd stage) of the antigenic complex in neutral and acid formamide*

		Properties of fraction			Components isolated from fractions after acid hydrolysis			
					Polysaccharide		Polypeptide	
Reagent	Fraction	$[\alpha]_{5461}$	Aqueous solution	N %	%	N %	%	N %
Neutral formamide	A (precipitate)	+40°	Strongly opalescent	4·8	56	1·6	28	8·0
	B (supernatant)	+86°	Clear	—	82	1·5	10	7·6
Acid formamide	C (precipitate)	+30°	Strongly opalescent	6·5	40	1·8	45	11·6
	D (supernatant)	+90°	Clear	2·2	76	1·7	3	4·8

contained as much as 45 % of polypeptide, whereas the more soluble material D contained only 3 % of this component.

The exact number of times the material must be treated with formamide to give a desired result must vary with the conditions employed. In most experiments a 2 % solution of the preparation in anhydrous neutral or acid formamide was kept for an hour at room temperature or several hours at 1–3° before it was fractionated with cold absolute alcohol. The properties of some of the various products arising from the original antigenic complex by the dissociating action of formamide are given in Table 4.

Table 4. *The properties of the products derived from the original antigenic complex*

				Antigenic activity in the rabbit as measured by *Bact. dysenteriae* (Shiga)*		
Material	Components	$[\alpha]_{5461}$	N %	Agglutinins	Precipitins	Haemolysins
Primary extraction product	Phospholipin Polysaccharide Polypeptide	+55 ±5° †	3·8–4·2	+ + +	+ + +	+ + +
Formamide dissociation product	Polysaccharide Polypeptide	+65 ±5° †	4·0–4·4	+ + +	+ + +	+ + +
Tryptic digest of primary extraction product	Polysaccharide Phospholipin	+60 ±5° †	2·2‡	0	0	0
Dissociation or acid hydrolysis product	Polysaccharide	+106° §	1·7	0	0	0
Do.	Polypeptide	−48° ‖	11·6	0	0	0
Do.	Phospholipin	+12° ¶	1·9	0	0	0

* After 3 doses of 0·01 mg.
‡ This specimen contained 4 % polypeptide component.
‖ In 0·1 *N* NaOH.
† In formamide (M.P. 1–2°).
§ In water.
¶ In pentane.

The action of trypsin on the antigenic material

In contrast with the result of an earlier experiment [Morgan, 1937], which suggested that the primary extraction product was antigenically active after treatment with trypsin at *p*H 8·4, later experiments, in which larger quantities of trypsin and longer digestion times have been employed, indicate that in the

earlier experiment the hydrolysis was incomplete and that some unchanged antigen remained. The removal of most of the polypeptide component from the polysaccharide-polypeptide-phospholipin complex was attained as follows. A 1 % solution (50 ml.) of the antigenic material was mixed with an equal volume of 0·5 M Na_2HPO_4 buffer at pH 8·4 and treated at 37° with four separate 25 mg. portions of a commercial trypsin preparation during 48 hr. The opalescence of the antigen solution diminished somewhat during the incubation. The solution was then dialysed against distilled water to remove sodium phosphate, centrifuged to remove insoluble material and finally, after concentration to a small volume, shaken with chloroform. The aqueous solution after centrifuging was dialysed at 0° for several days against frequent changes of distilled water, evaporated to a small volume and finally taken to dryness *in vacuo* after freezing the solution at −20°. The material thus obtained consisted of a fibrous white mass which readily dispersed in water to give an opalescent solution. The material forms a stable, colloidal solution but can be thrown down as a deposit when the solution is centrifuged for 60 min. at 4000 r.p.m. in an angle-centrifuge. The polysaccharide-phospholipin complex prepared in this manner usually contains 2–3 % of the polypeptide component and possesses only weak antigenicity. The slight immunological activity, however, is most probably due to the 2–3 % of polypeptide component which remains in the complex. The phospholipin component cannot be removed from the polysaccharide-phospholipin complex by extraction with alcohol-ether mixture or chloroform. The complex, which showed in one preparation $[\alpha]_{5461} + 60 \pm 3°$ (c, 0·5 in formamide), was readily split into its two principal components by the action of 0·1 N acetic acid at 100° for 45 min. and by the application of the standard technique polysaccharide ($[\alpha]_{5461} + 106°$ N, 1·7 %) and phospholipin (N : P, 1 : 1) components were subsequently isolated. In one specimen of polysaccharide-phospholipin hydrolysed, the polysaccharide and the phospholipin components represented 65 and 15 % respectively. The polysaccharide-phospholipin material can also be split into its components by simple solution in neutral formamide at 0° and subsequent precipitation by cold alcohol. The dissociation of the components under these conditions appears to be complete since the pure specific polysaccharide can readily be isolated after a single treatment with formamide.

The action of trypsin on the polysaccharide-polypeptide complex

A specimen (40 mg.) of antigenic material obtained by the action of neutral formamide on the primary extraction product and which yielded 60 % polysaccharide and 21 % polypeptide on acid hydrolysis was dissolved in distilled water (4 ml.) and 0·2 M Na_2HPO_4 (2 ml.) was added. The opalescent solution was then treated with commercial trypsin (8 mg.) dissolved in water (0·8 ml.) and incubated at 40°. In less than 1 min. the solution became quite clear but incubation was continued for 24 hr. The material was then dialysed in a small cellophane bag against frequent changes of distilled water for several days until the diffusate gave no test for inorganic phosphate thus indicating the complete removal of the buffer. The solution after dialysis was thoroughly shaken with chloroform which caused the non-diffusible portion of the trypsin preparation and a part of the polysaccharide to separate as an interfacial layer which could be subsequently removed by centrifuging. The clear aqueous supernatant fluid was then concentrated. After a further reprecipitation from aqueous solution by acetone a specimen of polysaccharide ($[\alpha]_{5461} + 98°$; N, 1·5 %) was obtained.

The reversibility of the formamide dissociation of the antigenic polysaccharide-polypeptide complex

The polypeptide component prepared by acetic acid (1 %) hydrolysis of the polysaccharide-polypeptide complex and subsequently purified by repeated precipitation from alkaline solution by dilute HCl and trituration with absolute alcohol is only slightly soluble in formamide or in a solution of polysaccharide in formamide. On the other hand a preparation of the polypeptide when freshly precipitated from solution in alkali by acid is still insoluble in formamide but dissolves readily in formamide in the presence of the specific polysaccharide. The utilization of this property has enabled an artificial polysaccharide-polypeptide complex to be prepared. A specimen (60 mg.) of polysaccharide prepared by formamide dissociation of the natural polysaccharide-polypeptide complex was dissolved in 2·5 ml. formamide (M.P. +2°). The clear viscous solution was added to a carefully purified sample (40 mg.) of polypeptide which was in the form of a water-moist centrifuged deposit. The polypeptide dissolved immediately on stirring the mixture and a clear viscous solution was obtained. This was kept at room temperature for 2 hr., diluted with an equal quantity of formamide and kept at 35° for 2 hr. longer, in order to allow the system to attain equilibrium. The clear solution was then cooled to 0° and absolute alcohol (also at 0°) added to yield an overall concentration of 75 %. A precipitate formed and was collected by centrifuging. This deposit was immediately taken up in 10 ml. of distilled water and it remained as a stable opalescent suspension during several days' dialysis at 0° against frequent changes of distilled water. A small amount of material, presumably the larger particles, was removed by centrifuging at 3500 r.p.m. in an angle-centrifuge for 20 min. The artificial complex was obtained in the form of a white fibrous mass by evaporating the frozen solution *in vacuo*. A part of the preparation was heated for 1 hr. at 100° with 1 % acetic acid and under these conditions it gave rise to a flocculent precipitate of the polypeptide component in the same manner as occurs with the natural complex.

The artificial complex and the original polysaccharide and polypeptide components used in the experiments were tested for antigenicity in rabbits. A course of 6 doses, each of 0·02 mg., was given intravenously and the animals were bled after 6 days. Agglutination and precipitation tests showed that the polysaccharide and polypeptide failed to induce the formation of specific "Shiga" immune bodies, whereas the artificial complex gave rise to an immune-serum that showed agglutination to a titre of 1 in 320 with *Bact. dysenteriae* (Shiga) and gave distinct precipitation with 1 : 10,000 and 1 : 100,000 dilutions of the specific polysaccharide. The immune-serum also gave a positive precipitin reaction when mixed with an equal volume of a 1 : 1000 dilution of the polypeptide component. These interesting results immediately suggest many further investigations, and experiments are already being undertaken to determine whether the specific polysaccharides of other organisms can be combined in this manner with the "Shiga" polypeptide component to yield an antigenic complex.

Discussion

Evidence is given that the primary extraction product which has been isolated from the "smooth" strain of *Bact. dysenteriae* (Shiga) consists essentially of a homogeneous molecular complex which is able to induce the formation of antibacterial immune-bodies similar to those produced by injection of the intact

organism. This material has been used for further chemical and physical investigation.

Gentle acid hydrolysis of the antigenic material yields a polypeptide-like component, a polysaccharide and a phospholipin complex. The amounts of these constituents isolated make up altogether 80–90 % of the weight of the original antigenic material and thus indicate that a fourth component, if it exists, must be quite small in amount. The polysaccharide component is built up from *d*-galactose, *l*-rhamnose and *N*-acetylglucosamine units whereas from the phospholipin molecule oleic acid, palmitic acid and α-glycerophosphoric acid have been obtained. The nitrogenous constituent of the phospholipin has not been isolated. The polypeptide-like component is an acidic, amorphous substance containing 11·6 % N, and tyrosine (8 %) and arginine (5·5 %) have been identified by colorimetric methods as constituent amino-acids. After hydrolysis with *N*-HCl-10*N* formic acid mixture nearly 50 % of the total N of the polypeptide is in the form of free amino-groups (Van Slyke). A more detailed account of this important component of the natural antigenic complex will be given in a later paper.

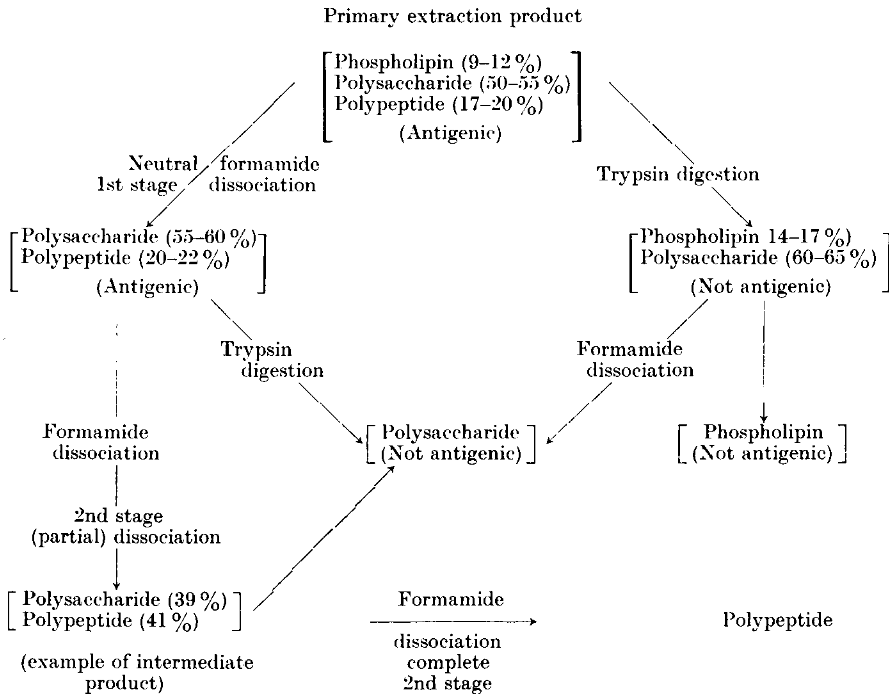
The hydrolysis experiments reveal the existence within the antigenic complex of some easily hydrolysable phosphate (about 10 % of the total P) which is presumably in organic combination but which is ultimately found in the form of inorganic phosphate in the alcoholic supernatant fluid remaining after the three principal components have been removed. This residual material (Fraction IV) may possibly contain a further component but it has not as yet been examined in any detail.

Although antigenic material has been isolated from various organisms and studied by a variety of methods there has appeared as yet no simple, systematic technique whereby it has been possible to break down the antigenic complex step by step and investigate the chemical, physical and immunological properties of the resulting degradation products. In this paper a method whereby this may be accomplished is described. The method is based on the dissociating action of the highly polar solvent, formamide. Further work will certainly modify and improve the technique to suit the nature of the material under investigation better, but it seems probable from our results that the use of highly polar organic solvents opens up a new method of attacking the problem of the constitution of certain naturally occurring hydrophobic substances of complex composition, of which bacterial antigens are but a single example. In this connexion it is of interest to recall the reported dissociation of native horse haemoglobin into molecules of half the normal molecular weight by means of aqueous formamide [Steinhardt, 1938].

The degradation of the natural antigen by means of neutral or acid formamide or trypsin into simpler complexes is shown diagrammatically in Fig. 1 where the various steps in the process and the approximate compositions of the resulting products are clearly set out. Different preparations of antigen have, under the experimental conditions described, given rise to material that differs in composition within the limits shown. This variation in composition is to be expected in view of the ease with which the components of the natural antigen are dissociated by the strongly polar solvent and it is clear that the separation of the antigenic material into products with the composition given will only be attained if the experimental conditions follow closely those described in the earlier part of this paper.

The first stage in the formamide dissociation process, the removal of phospholipin, is a relatively simple one. It appears that in formamide solution the dissociation of the phospholipin from the remainder of the complex is almost

complete and in consequence one precipitation with alcohol brings down the bulk of the material which is then found to be associated with only 1–2 % of the phospholipin in place of the original 9–12 % in the natural antigenic complex. A repetition of the process yields an antigenic material in which no phospholipin is detectable. The polypeptide component, however, is only partially dissociated from the polysaccharide in formamide solution under the conditions described, for if the phospholipin-free material is dissolved in neutral or slightly acid



Note. All polysaccharide percentages given are for material *isolated* and must be considered 10–20 % too low.

Fig. 1.

formamide and fractionated with alcohol insufficient in amount to precipitate completely the whole of the complex, the less soluble material which is first thrown out of solution contains a greater relative proportion of polypeptide than does the later material which is precipitated with increasing alcohol concentration. By the successive removal in this manner of the most soluble material—the polysaccharide—a polysaccharide-polypeptide complex may be obtained which contains as much as 50 % of the later component. Material that contains as little as 2–3 % of polypeptide has been isolated from the more soluble fraction and preparations containing 5–10 % of this component have been tested and found to be antigenic. The polysaccharide remaining in the final alcoholic formamide possesses properties very similar to that of the polysaccharide isolated by direct treatment of the micro-organisms with hot acetic acid [Morgan, 1936] except that it dissolves less readily in water, gives rise to a

solution of high viscosity and contains about 0·5% of phosphate in organic combination. The polysaccharide isolated by either method, however, is not antigenic. In view of the mild conditions of treatment involved in the alcoholic formamide fractionation process it is unlikely that the polysaccharide has been altered chemically during its dissociation from the polypeptide component. It appears probable, therefore, that the loss of antigenicity of the complex is associated in some manner with the loss of the polypeptide and not with any chemical change in the polysaccharide molecule. An essential function of the polypeptide, which is itself but weakly antigenic, may be to create a mechanism whereby intermolecular association is able to occur that will ultimately give rise to a large hydrophobic colloidal particle. A complex of this nature which is not readily eliminated by the animal body would presumably possess antigenic properties. A mechanism which it is thought might explain the dissociating action of formamide has been suggested by Steinhardt [1938] who considers it likely that formamide functions as a dissociating agent for proteins because it possesses an amide bond similar to that which occurs repeatedly as the peptide link in proteins and which is responsible for a considerable measure of polar association between the polypeptide chains. Steinhardt suggests that the formamide becomes involved in the competition for the peptide bonds of adjacent chains and thus weakens or eliminates completely the tendency of the bonds to associate. It is of interest to recall that the dialysis of a clear solution of the antigenic material in formamide gives rise to strongly opalescent aqueous solutions, presumably owing in part to the reaggregation of the dissociated components since the product that can be subsequently isolated is similar in general chemical and immunological properties to the original material. A number of experiments have been carried out with the object of ascertaining whether it is possible to reconstitute the original antigenic complex from the antigenically inactive components. The experiments have not been described in any detail owing to their preliminary nature but it has been observed that when the essential components of the antigenic complex—the polysaccharide and polypeptide—are mixed together in formamide solution and the resulting complex is subsequently isolated by an appropriate technique, then this complex has definite antigenic properties and gives rise to specific *Bact. dysenteriae* (Shiga) agglutinins and precipitins. From the immunological standpoint this result indicates that after the complete dissociation of the components of the system, their subsequent re-aggregation, which results from the removal of the dissociating solvent, occurs in a regular manner.

The method used for the extraction of the antigenic material from the bacterial cell suggests that the primary extraction product is in reality a degraded form of the antigenic complex as it exists in the living micro-organism. This suggestion would offer an explanation for the observed fact that commercial trypsin does not attack the antigen when it is present in the intact bacterial cell whereas preparations of the antigenic complex isolated by means of diethyleneglycol are slowly attacked. If one may judge from the results obtained by the use of formamide, extraction of the antigenic material with diethyleneglycol will induce some measure of dissociation which will result in the loss of part of the original antigenic complex. Diethyleneglycol, however, with its low dielectric constant would almost certainly be less active as a dissociating medium than the strongly polar formamide which possesses a remarkably high dielectric constant. In any event, the antigenic material after isolation is slowly attacked by trypsin and is thereby almost completely deprived of its polypeptide-like component. Furthermore, the phospholipin-free antigenic material, which represents a

further stage in the degradation of the antigenic complex, is quite readily attacked by the enzyme preparation with destruction of the polypeptide component and the loss of antigenic properties. A similar difficulty in removing a polypeptide-like component which is bound to polysaccharide has been reported recently by Hewitt [1939] who attempted to isolate the polysaccharide components of seroglycoid and globoglycoid by means of both peptic and tryptic hydrolysis. His results show that even after one treatment with pepsin followed by three treatments with trypsin the carbohydrate represented only about 50% of the total carbohydrate-polypeptide complex. It would be of interest to know whether these glycoproteins can be separated more readily into their polysaccharide and protein components by means of formamide.

There is additional evidence that the antigenic complex is changed in some manner during the extraction process. The original antigenic material shows an appreciable solubility in anhydrous diethyleneglycol, whereas after isolation from this solvent, a process that can be accomplished by simple dialysis and evaporation at low temperature, the material no longer shows any definite solubility in diethyleneglycol. It seems probable, therefore, that the insolubility of the isolated material is due to loss of a part of the complex as a result of the dissociating action of the diethyleneglycol.

It has been observed that the opalescence of an aqueous solution of the polysaccharide-polypeptide complex in phosphate buffer at pH 8·4 disappears completely a few seconds after the addition of trypsin. At this stage the polypeptide is apparently liberated from its union with the polysaccharide but it is not split into its constituent amino-acids as can be readily shown by acidifying the reaction mixture, which results in the immediate precipitation of the insoluble polypeptide component. The rapid dissociation of the polypeptide from its combination with the polysaccharide recalls the recent observations of Pope [1938; 1939] on the disaggregating action of enzymes on antitoxic pseudoglobulin, whereby the complex molecule is split into two different components with no demonstrable degradation into the constituent amino-acids. This interesting phenomenon is being further investigated.

By the action of neutral formamide it has been possible to remove the phospholipin component from the primary extraction product and obtain a substance that consists essentially of a complex of polysaccharide and a polypeptide-like material. This substance is strongly antigenic and gives rise to specific "Shiga" heterophile immune-body as well as "Shiga" agglutinins and precipitins. In view of the general belief that phospholipins play an important part in the antigenic structures which lead to the production of heterophile haemolytic antibody it is of considerable interest to find that the antigenic complex after treatment with neutral formamide, which removes the phospholipin component, remains strongly antigenic and induces the formation of "Shiga" haemolytic immune-body qualitatively identical with that produced by means of the intact micro-organism. This result, however, is in complete agreement with the evidence brought forward by Meyer & Morgan [1935] and Meyer [1938], that the specific polysaccharide of *Bact. dysenteriae* (Shiga) possesses the property of neutralizing the haemolytic action of "Shiga" heterophile antibody on sheep red cells, in addition to its property of combining with the homologous antibacterial immune-substance as indicated in agglutination, precipitation and complement-fixation tests. It has also been shown that the polysaccharide-phospholipin complex which arises from the prolonged action of trypsin on the antigenic material is most probably incapable, when completely free from the polypeptide component, of inducing in the rabbit the formation of "Shiga"

heterophile immune-body. These two pieces of evidence taken together indicate that the phospholipin component plays no significant part in the production or neutralization of "Shiga" haemolytic antibody.

An attempt has been made to find out whether the antigenic material isolated by means of trichloroacetic acid according to the technique of Boivin *et al.* [1935] is similar in general properties and behaviour to the material obtained by extraction with diethyleneglycol. The results are inconclusive, for although the polysaccharide component (54 %) and pentane-soluble phospholipin (6 %) appear to be identical, the polypeptide could not be isolated by the usual technique. The preparation was found to contain other nitrogenous material and it has not as yet been possible to separate a polypeptide component identical with our own. The antigenic material extracted by means of trichloroacetic acid, however, gave rise to a phospholipin-free product on treatment with formamide which was found to be antigenic when tested in the rabbit.

Although it is perhaps beyond the scope of the present communication to suggest the nature of the intermolecular forces that bind together the various component molecules which make up the natural antigenic complex, it would appear from the results that have been obtained that these bonds are not of the covalent type and that in consequence stereochemistry of the linkages and constant molecular composition for the antigenic complex are not to be expected. It must remain, however, for future work to decide whether the association of the various component molecules of the antigenic complex is due to polar forces alone, to the "hydrogen" or to the "hydroxyl" bond or to some other as yet unknown mechanism.

As a result of some earlier experiments in which antigenic material prepared by three different methods was examined, the idea that perhaps a bacterial antigen was not a chemical compound of fixed composition gradually developed. It appeared conceivable that a bacterial antigen as it existed in the intact bacterial cell was not a single chemical compound of rigid composition but consisted of a labile molecular aggregate possessing an essential component—such as a polysaccharide—of definite chemical structure and of fixed composition which determined the strict immunological specificity of the antigen, together with other loosely bound constituents which endowed the essential component with antigenic properties. It seemed probable that only part of these physically associated molecules were necessary for full antigenic activity and that certain of these constituents could, therefore, be dissociated from the labile complex during the isolation and purification of the antigen without producing more than a moderate reduction in the antigenic capacity of the active material. It was believed that further and more thorough physical disaggregation of the associated constituents would ultimately lead to complete loss of antigenicity. Some evidence in support of this conception of antigenic structure is given in this paper.

The work that has been described represents an attempt to learn something of the general structure of a bacterial antigen and the results have enabled a conception of antigenic structure to be developed. It is not believed, however, that the conclusions are in any way final. Indeed much further evidence must be collected before they can be completely accepted even in their present form, although the experimental facts given would seem sufficient to justify the temporary acceptance of the conception as a working hypothesis. It would appear as a result of similar experiments carried out with antigenic material isolated from specific strains of *Bact. typhosum* that the polysaccharide-polypeptide-phospholipin type of complex may be of fairly wide occurrence as a basic structure in certain bacterial antigens.

Summary

1. The dominant antigenic complex associated with the "smooth" form of *Bact. dysenteriae* (Shiga) is shown to consist of three major components—a polysaccharide, a phospholipin and a polypeptide-like substance. Other minor components may also be present.

2. A method is described for the stepwise degradation of the antigenic complex which in part depends on the dissociating action of formamide.

3. A conception of antigenic structure is suggested which is supported by certain of the experimental results.

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