59. THE HYDROLYTIC DEGRADATION OF THE ANTIGENIC COMPLEX OF *BACT. TYPHOSUM* Ty2

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THE antigens of a number of Gram-negative micro-organisms have been isolated in a relatively pure condition and apparently unchanged in their specific immunological properties by various methods in the work of Boivin *et al.* [1933; 1934; 1935; 1937], Raistrick & Topley [1934], Topley *et al.* [1937], Morgan [1937], Morgan & Partridge [1940], and Miles & Pirie [1939, 1, 2, 3]. By use of the trichloroacetic acid method of extraction of Boivin *et al.* and the tryptic digestion method of Raistrick & Topley, Freeman *et al.* [1940] obtained the antigens of *Bact. typhosum* and *Bact. typhi-murium* and showed that by growth of the organisms in a synthetic medium various sources of contamination were eliminated.

The present communication describes a more detailed investigation of the chemical nature of the antigenic fractions obtained from *Bact. typhosum* Ty2 by the above methods.

The Ty2 strain of *Bact. typhosum* and its isolated antigen react with both O and Vi antisera; this is regarded by some authors as being due to the presence of two chemically and immunologically distinct entities, the O and Vi antigens. Boivin & Mesrobeanu [1938, 2] claim to have separated these components by precipitating the Vi antigen with uranyl acetate but attempts to repeat this work by the present authors have so far proved unsuccessful. Malek [1939] was also unable to separate the O and Vi component antigens by means of uranyl acetate. Meyer [1938] has, however, stated that the O and Vi antigens of an O+Vi strain of *Bact. typhosum* are distinct molecular entities which can be separated by their precipitation reactions towards specific antisera. Thus, whilst in the investigation to be described the antigen of *Bact. typhosum* Ty2 has been regarded as an individual molecule, the possibility of its consisting of component O and Vi antigenic molecules must not be overlooked.

EXPERIMENTAL

The bulk of the starting material in this investigation was prepared from the dried cells of *Bact. typhosum* Ty2, grown on a synthetic medium, by tryptic digestion as described by Freeman *et al.* [1940].

Properties of the antigenic complex

The tryptic digest antigen of *Bact. typhosum* Ty2 dissolves readily in water giving opalescent solutions even when the concentration of antigen is as low as

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0·4 %. The bulk of the substance remains in solution after 30 min. centrifuging at 15,000 r.p.m. The antigenic complex also dissolves readily in formamide and formic acid giving solutions which are practically free from opalescence, but it is insoluble in glacial acetic acid, dioxan and in concentrated aqueous solutions of phenol. Qualitative tests have been made on 0·5 ml. quantities of a 0·4 % aqueous solution of the antigen; the test solutions were centrifuged at about 2000 r.p.m. for 15 min. and 0·5 ml. of the opalescent supernatant solution was mixed with 0·5 ml. of the reagent. Acid potassium permanganate was slowly reduced by the antigenic solution in the cold and more rapidly on warming. Addition of uranyl acetate solution produced an immediate flocculent precipitate, whilst phosphotungstic acid in 5 % H_2SO_4 gave a precipitate on standing and neutral lead acetate an increased opalescence on standing. No precipitates were obtained on addition of Esbach's reagent, 10 % trichloroacetic acid solution, salicylsulphonic acid or tannic acid. Tyrosine was present as indicated by positive Millon's, xanthoproteic, α-nitroso-β-naphthol and Ehrlich's diazo reactions. The Adamkiewicz and aldehyde reactions and Ehrlich's *p*-dimethylaminobenzaldehyde test gave negative results showing the absence of tryptophan. The sodium fusion test and boiling of the antigen with strong NaOH and addition of lead acetate proved the absence of sulphur. A positive Sakaguchi reaction for arginine was obtained. The antigenic solution also gave positive biuret and ninhydrin reactions, indicating the presence of peptide linkages and amino-groups respectively. The substance gave a very strong Molisch's reaction but did not reduce Fehling's solution. Pentose residues (furfuraldehyde, Tollen's phloroglucinol and Bial's tests), ketose (Seliwanoff's and Foulger's tests) and glucuronic acid residues (Tollen's naphthoresorcinol reaction) were found to be absent. The solution gave no iodine reaction.

The nitrogen content and reducing sugar value of the antigenic preparations varied within narrow limits from batch to batch and a typical sample gave: N, 7·2 %; reducing sugar value, 32 %; $[\alpha]_{5461} = +104°$ (in aqueous solution, $c=1$), $[\alpha]_D^{17°} = +69°$ (in formamide solution, $c=1$), and $[\alpha]_D^{13°} = +68°$ (in formic acid solution, $c=1$); non-volatile ash, 12·2 %; organic P, 3·0 %; inorganic P, 1·2 % (N/organic P ratio, 5·0). Determination of amino-N by Van Slyke's method showed that 0·6 % of N was present as amino-groups.

The tryptic digest antigen was toxic for mice, having an average lethal dose of 0·3 mg. [Freeman *et al.* 1940]. Immunizing experiments similar to those described by Topley *et al.* [1937] have been carried out on practically every batch of antigen, and the results have shown that the substance possesses a high degree of immunological activity comparable with that of the original dried bacterial cells.

Acetic acid hydrolysis of the antigenic complex

The tryptic digest antigen was dispersed by shaking with 0·1*N* acetic acid to give a 1 % solution, which was centrifuged at 15,000 r.p.m. for 2–3 hr. to remove undissolved material ('undissolved residue'). The supernatant solution which was slightly opalescent was hydrolysed at 100° on the boiling water bath for 12 hr. On heating, the solution first clarified a little (30 min.), but after 2 hr. it became very opalescent and an insoluble substance began to flocculate out. After about 8 hr. heating the flocculation appeared to be complete and there was no further deposition of precipitate and no change of optical rotation during the last 2 hr. The insoluble material ('hydrolysis precipitate') was centrifuged off, washed with a little water and dried *in vacuo* over phosphorus pentoxide.

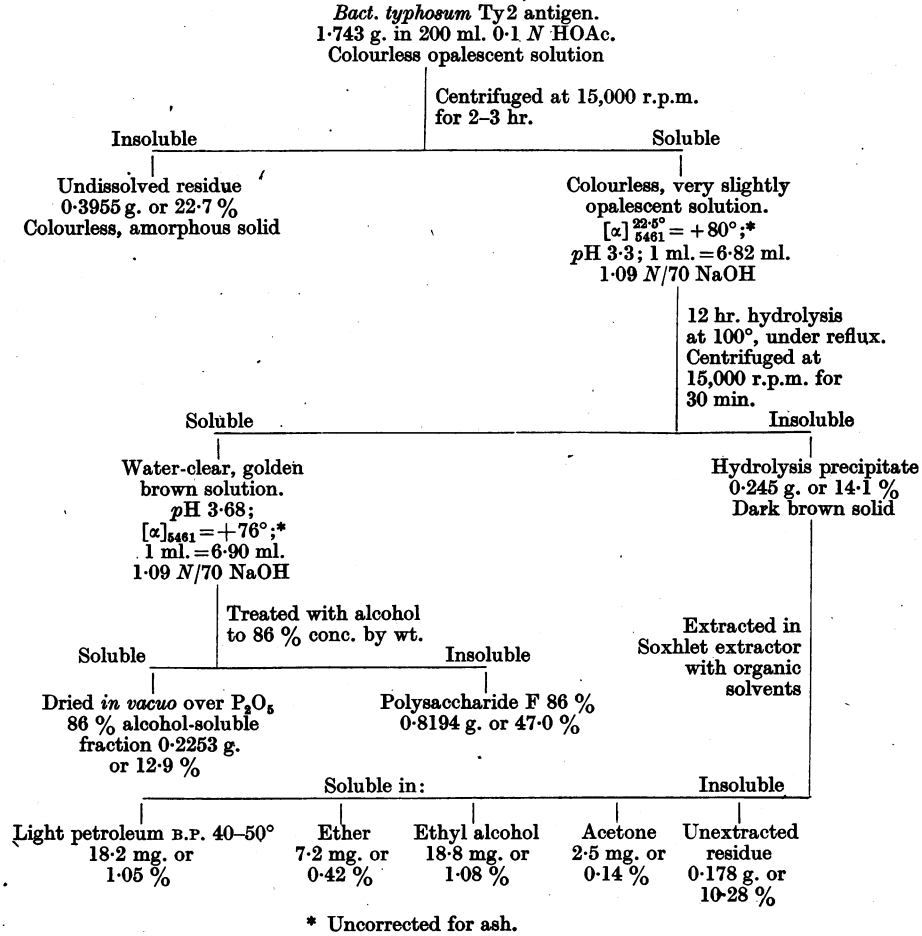
The dried hydrolysis precipitate was then exhaustively extracted in a Soxhlet extractor, successively with:

(1) Light petroleum, B.P. 40–50°,
(2) Pure anhydrous ether,
(3) Absolute ethyl alcohol, and
(4) Acetone.

The solvents were evaporated from the extracted lipid components, which were stored *in vacuo*. The remainder ('unextracted residue') was dried *in vacuo* over phosphorus pentoxide.

The acetic acid supernatant solution, containing the soluble hydrolysis products, was water-clear and golden brown in colour; it was treated with sufficient absolute alcohol to bring the alcoholic concentration to 86%, by weight, giving a precipitate rich in polysaccharide ('Polysaccharide F 86 %'). This

Table 1. *0·1N acetic acid hydrolysis of* Bact. typhosum *Ty2 'tryptic digest antigen'*



* Uncorrected for ash.

Total recovery of fractions = 96 %.

fraction was washed twice with absolute alcohol, twice with ether and dried *in vacuo* over phosphorus pentoxide.

The alcoholic supernatant solution and washings of polysaccharide F 86 % were evaporated to dryness *in vacuo*, giving a brown-coloured sticky residue: '86 % alcohol-soluble fraction'.

The fractionation is summarized in Table 1. The fractions thus isolated have been submitted to qualitative and quantitative examination; Kjeldahl N, non-volatile ash, reducing sugar value, specific optical rotation, total and inorganic P, amino-N and acidity were determined. The N, ash, reducing sugar value and specific rotation determinations were carried out as described by Freeman *et al.* [1940]. Total P determinations were made on 0·5–1·0 mg. quantities of substance by Stewart & Hendry's [1935] modification of the Fiske & Subbarow [1925] method. Inorganic P determinations were made on 2–5 mg. quantities of substance by the Fiske & Subbarow method. Organic P has been taken as the difference between total and inorganic P, and equivalent N/organic P ratios have been calculated on this basis. The amino-N content of the soluble fractions has been determined on 10 mg. samples by the Van Slyke method. Acidity determinations were made by titrating 5–10 mg. quantities dissolved in a few ml. water with 0·014*N* NaOH using phenolphthalein as indicator. Except where otherwise stated analytical results are quoted on an ash-free basis. The results obtained for the fractions isolated by partial acetic acid hydrolysis of the antigenic material are summarized in Table 2.

Table 2. *Analytical data of fractions isolated from* Bact. typhosum *Ty2 antigen* (1·74 g.)

	Un-dissolved residue	Un-extracted residue	Total lipid	Poly-saccharide F 86 %	86 % alcohol-soluble fraction
Weight (g.)	0·395	0·178	0·047	0·819	0·225
Yield (%)	22·7	10·3	2·7	47·0	12·9
N (%)	8·9	6·0	0·6	3·6	13·4
Non-volatile ash (%)	7·4	5·0	—	20·2	5·4
Reducing sugar value (%)	28	10	—	55	19
$[\alpha]_{5461}$	—	—	—	+142°	+7°
Inorganic P (%)	—	—	—	3·0*	0·2*
Organic P (%)	4·1	2·2	0·5	1·6	0·6
N/org. P ratio	4·7	6·0	2·6	4·8	48
Amino-N (%)	—	—	—	0·5	3·0
Acidity ml. *N* NaOH/g.	—	—	—	0·55*	1·38*
	* Uncorrected for ash.				
% Yield on total dissolved matter	—	13·3	3·5	60·8	16·7

Further samples of antigenic material were hydrolysed under slightly different conditions with similar results. Soru & Combiesco [1940] report fission of the *Bact. typhosum* O-antigen, prepared by trichloroacetic acid extraction of the organisms, by hydrolysis with 0·04*N* acetic acid for 45–60 min. at 100°. Some of our hydrolyses were made with 0·1*N* acetic acid for 2 hr., and in one experiment a trichloroacetic acid extract of the bacteria, 'Boivin antigen F 68 %', was hydrolysed with 0·04*N* acetic acid for 60 min. at 100°. In these cases in which a shorter period of hydrolysis was used the flocculation of the hydrolysis precipitate was incomplete and this fraction could not be completely precipitated on centrifuging. Addition of alcohol to about 30 % concentration, by weight, was required to complete the flocculation of the hydrolysis precipitate. (The polysaccharide component is completely precipitated between the limits

Table 3. *Hydrolysis of further batches of* Bact. typhosum *Ty2 antigen with dilute acetic acid*

Conditions of hydrolysis, etc.		Hydrolysis precipitate	Polysaccharide F 86 %	86 % alcohol-soluble fraction
1. 3·644 g. tryptic digest antigen dissolved in 335 ml. 0·1 *N* acetic acid and hydrolysed at 100° for 12 hr.	Yield g. ...	0·493	1·937	0·498
	% of antigen ...	13·6	53·2	13·7
	Properties ...	Main fraction after solution in *N* NaOH and repptn. by HCl and extraction with organic solvents gave: N 8·2 %, R.V. 10 %, ash 2·0 %, P (total) 2·4 %	After two fractionations between 42 % and 85 % conc. of alcohol, by weight, gave: N 2·2 %, R.V. 68 %, ash 6·9 %	P (total) 1·9 % Ash 9·0 % R.V. 19 % N 14·3 % Amino-N 1·8 %
2. 5·07 g. tryptic digest antigen dissolved in 500 ml. 0·1 *N* acetic acid and hydrolysed at 100° for 12 hr.	Yield g. ...	1·122	2·602	1·157
	% of antigen ...	22·8	53·1	23·6
3. 4·54 g. tryptic digest antigen dissolved in 500 ml. 0·1 *N* acetic acid and hydrolysed at 100° for 2 hr.	Yield g. ...	1·1576	2·721	0·2926
	% of antigen ...	25·5	59·9	6·4
4. 0·598 g. 'Boivin antigen F 68 %' dissolved in 112·5 ml. 0·04 *N* acetic acid and hydrolysed at 100° for 60 min.		Ppt. on centrifuging / Alcoholic ppt. F 50 %	(F 50–85 %)	—
	Yield g. ...	0·0156 / 0·3092	0·1828	—
	% of antigen ...	2·6 / 51·7	30·6	—
	Properties ...	N 6·0 % / N 6·4 % Ash 14·8 %	R.V. 64 % Ash 8·1 % N 2·57 % P (total) 1·8 %	—

of 40–85 % concentration of alcohol, by weight, but does not precipitate to any appreciable extent at 30 % alcoholic concentration.) These experiments are summarized in Table 3.

The undissolved residue which may be separated from solutions of the antigenic material by high-speed centrifuging, as in the original hydrolysis experiment, is to be regarded as an insoluble or 'denatured' modification of the antigen, which tends to pass into an insoluble form on drying and storage. This fraction, therefore, has not been removed in the later experiments but submitted to hydrolysis with the bulk of the antigenic material.

The experiments 1–3, summarized in Table 3, confirm the results of the original hydrolysis experiment (Tables 1 and 2); when the time of hydrolysis is shortened to 2 hr. (exp. 3) the separation into soluble and insoluble fractions was much less definite and the hydrolysis precipitate was not completely flocculated until an alcoholic concentration of 32 % (by weight) had been reached. 6 % of insoluble substance was removed by centrifuging at 3000 r.p.m. on an angle centrifuge for 4 hr., leaving a very opalescent supernatant solution, from which the remaining 19·5 % of insoluble matter was precipitated by an alcoholic concentration of 32 % (by weight) leaving a water-clear supernatant solution. In this experiment the 86 % alcohol-soluble fraction was also much smaller in amount. In exp. 4, in which a trichloroacetic acid extract of *Bact. typhosum* Ty 2 'Boivin antigen F 68 %') was hydrolysed under the conditions employed by Soru & Combiesco [1940], there was an even greater difficulty in flocculating the insoluble fraction. Only 2·6 % could be precipitated by centrifuging (3000 r.p.m. on an angle centrifuge for 3 hr.), and alcohol to 50 % concentration (by weight) had to be added in order to complete the flocculation of the very opalescent supernatant solution. This produced 52 % of substance, which was probably incompletely hydrolysed. A crude polysaccharide, alcoholic fraction F 50–85 %, was obtained which was very similar in properties to the crude polysaccharide obtained on hydrolysis of the tryptic digest antigen.

The mean of the results obtained in exps. 1–3 and in the original hydrolysis experiment (Table 2) point to the conclusion that on mild hydrolysis the antigen of *Bact. typhosum* Ty 2 yields about 57 % of crude polysaccharide, 18 % of alcohol-soluble fraction and 20 % of hydrolysis precipitate, of which about 4 % (in terms of the original weight of antigen) is of a lipid nature. These four constituents will now be considered in fuller detail.

The polysaccharide component of the antigenic material

The crude polysaccharide was a water-soluble, almost colourless amorphous substance, which corresponded to about 57 % of the antigenic complex. As first precipitated it contained 3·6 % N, 1·6 % organic P and a considerable amount of ash. On hydrolysis with 2*N* HCl for 2 hr. in a sealed tube 55 % of reducing sugars were liberated (Hagedorn & Jensen). At this stage the polysaccharide had a small titratable acidity corresponding to 0·5 ml. *N* NaOH/g. and had a high specific rotation of about $[\alpha]_{5461} + 140°$. The polysaccharide dissolved readily in water, giving solutions which were water-clear. Solutions of the crude material failed to give a positive Millon's reaction or α-nitroso-β-naphthol test for tyrosine; the biuret reaction was also negative. Weakly positive ninhydrin tests and the Sakaguchi reaction for arginine indicated that small amounts of amino-acid residues remained as impurities in the polysaccharide. This was confirmed by the appearance of a trace of precipitate on addition of phosphotungstic acid in 5 % H_2SO_4 and of an opalescence on addition of neutral lead acetate. The polysaccharide gave a very strongly positive Molisch's reaction. It gave no coloration

with iodine and failed to reduce Fehling's solution. There was no evolution of furfuraldehyde on heating with 12 % HCl. Bial's reaction and Tollen's phloroglucinol test for pentoses were also negative as were the Seliwanoff test and Foulger's test for ketoses. Tollen's naphthoresorcinol test indicated the absence of uronic acid residues.

The purification of the polysaccharide by means of alcoholic and acetic acid fractionations or by preparation of the triacetyl derivative and subsequent deacetylation will be described in a later communication. An identical polysaccharide was obtained by treatment of the dried bacterial cells with dilute acetic acid at 100°. After purification the polysaccharide was obtained free from N and contained only traces of P (0·1–0·2 %). The purified substance had a reducing value of 1·1 % before hydrolysis, and on hydrolysis with 2*N* HCl for 2 hr. at 100° in a sealed tube gave 83 % of reducing sugars (Hagedorn & Jensen). It was highly dextrorotatory, $[\alpha]_D = +114°$ and $[\alpha]_{5461} = +140°$. Sulphur was absent and the ash content had been reduced to 1–2 %. On hydrolysis the polysaccharide yielded 3 % of volatile fatty acid (calculated as acetic acid), indicating the presence of acetyl residues in the molecule. (Found: C, 44·41; H, 7·12 %. $C_6H_{10}O_5$ requires C, 44·41; H, 6·22 %.)

The above-mentioned qualitative tests on the crude material indicate the absence of ketose, pentose and uronic acid residues from the polysaccharide molecule and repetition of the tests on the purified substance gave the same results, showing that such residues are either entirely absent or present only in very small amounts. On hydrolysis of the polysaccharide, derivatives of *d*-mannose, *d*-galactose and *d*-glucose have been isolated; these hexoses constitute the bulk of the polysaccharide molecule.

Our colleague, Dr A. Felix, has shown that solutions of the polysaccharide precipitate with 'pure' *Bact. typhosum* O-antiserum to a dilution of 1 in 15,000,000 of the polysaccharide, but not with 'pure' Vi-antiserum. The polysaccharide represents the O-hapten of the bacterium.

The polypeptide component of the antigenic material ('unextracted residue')

The fraction which is insoluble in water and organic solvents ('unextracted residue') constitutes about 16 % of the antigenic complex. In its crude state it is obtained as a brownish coloured solid which dissolves almost completely in 2*N* NaOH and is reprecipitated on acidification. Identification of constituent residues in the unhydrolysed substance is difficult owing to its insolubility. Although the ninhydrin reaction is negative, the presence of tyrosine and arginine is evident from positive xanthoproteic, Millon's and α-nitroso-β-naphthol tests, and the Sakaguchi reaction respectively. After partial hydrolysis with mineral acid a positive biuret reaction is obtained.

Fractionation of the hydrolysis precipitate. The dark greyish-brown hydrolysis precipitate obtained in exp. 1 (Table 3), and unextracted with organic solvents, was suspended in water and NaOH was added to *N* concentration. The bulk of the precipitate dissolved in the alkali to give a dark brown solution, leaving a small amount of undissolved residue which was removed by centrifuging. This undissolved residue was suspended in a few ml. of 2*N* NaOH when it showed anisotropy of flow and remained undissolved. It was recovered by acidification as a greyish-white precipitate. This was dried *in vacuo* over P_2O_5 and yielded 0·035 g. of 'hydrolysis precipitate (fraction insoluble in NaOH and HCl)'.

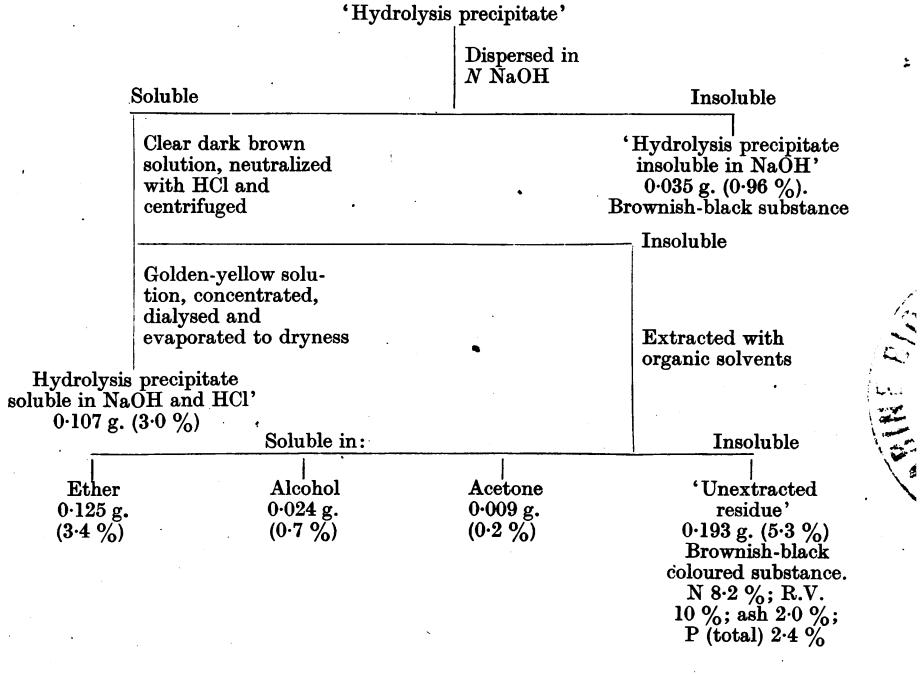
The clear brown alkaline solution containing the bulk of the hydrolysis precipitate was made slightly acid with HCl when a buff-coloured precipitate

of the acidic hydrolysis precipitate was obtained. The solid was centrifuged, taken up in a small quantity of 0·1 *N* NaOH in which it dissolved readily, and reprecipitated by neutralization with HCl; the precipitate was finally washed free from chlorides with distilled water.

The golden-yellow acid supernatant solution obtained on reprecipitation of the hydrolysis precipitate was neutralized and concentrated *in vacuo*. The concentrated opalescent suspension was dialysed in a collodion sac against frequent changes of distilled water for 24 hr. to remove sodium chloride, etc. and the contents of the sac were evaporated to dryness *in vacuo* giving 0·107 g. of a brown substance: 'Hydrolysis precipitate (fraction soluble in NaOH and HCl)'.

The bulk of the hydrolysis precipitate (i.e. soluble in NaOH and reprecipitated by HCl) was suspended in 20 ml. water and extracted with ether in a continuous liquid extraction apparatus for 40 hr. The ethereal solution on evaporation yielded 0·125 g. of a light brown waxy substance. The unextracted brown hydrolysis precipitate was centrifuged down and dried *in vacuo* over P_2O_5 yielding 0·290 g. of a brownish-black substance. This was extracted in a Soxhlet extractor with absolute ethyl alcohol for 24 hr., and 0·0238 g. of brown fatty material was recovered from the alcoholic solution. The unextracted residue was then extracted with acetone for 24 hr. in a Soxhlet extractor and 0·009 g. of lipid material was recovered from the acetone extract. The final unextracted residue consisted of 0·193 g. of a dark brown amorphous substance. The above fractionation is summarized in Table 4.

Table 4. *Fractionation of 'hydrolysis precipitate'*



The purified fraction obtained in the above fractionation contains N 8·2 %, P (total) 2·4 % and has a reducing value on hydrolysis with 2 *N* HCl for 2 hr. in a sealed tube at 100° of 10 % apparent glucose. The substance is acidic, being

readily soluble in dilute alkalis in the cold, but insoluble in water and dilute acids; it is so resistant to acid hydrolysis that it is only partially soluble on treatment with 2*N* HCl in a sealed tube for 2 hr. at 100°. The partial hydrolysate thus obtained gives an intense yellowish-red coloration with diazobenzene-sulphonic acid in alkaline solution (Ehrlich's diazo-reaction) and a positive α-nitroso-β-naphthol reaction indicating the presence of tyrosine. The ninhydrin reaction is also strongly positive at this stage.

Tryptic digestion of the 'unextracted residue'. The action of trypsin on the polypeptide-like fraction ('unextracted residue') has been investigated and the liberation of amino-acids during tryptic digestion followed by means of the Van Slyke method. 0·075 g. of the 'unextracted residue', prepared as described in Table 4, was freshly precipitated by acidifying an alkaline solution of the substance and then suspended in 15 ml. of borate buffer at *p*H 8·46. A brownish-coloured opalescent suspension was obtained. 0·003 g. commercial trypsin (Fairchild Bros. and Foster, New York) was added and the digest incubated at 37° for 14 days at *p*H 8·2–8·5, a further 0·003 g. trypsin being added after 2 days' digestion. 1 ml. portions of the digest were withdrawn at intervals and the amino-N estimated by the Van Slyke method. A blank determination was also made on a sample of trypsin which itself contained a small amount of amino-N. After 2 days' digestion only 7·3 % of the total N had been liberated as amino-N and 13·4 % after 14 days. The substance was therefore only slowly and very incompletely attacked by trypsin; its resistance to tryptic digestion is evident from the fact that after 14 days' exposure to the enzyme under these conditions an insoluble substance similar to the undigested compound was precipitated on acidification.

Acid hydrolysis of the 'unextracted residue'. Morgan & Partridge [1940] found that the polypeptide component of the *B. dysenteriae* (Shiga) antigenic complex was readily hydrolysed by 10*N* formic acid containing 3·6 % HCl. It has already been pointed out that the polypeptide-like fraction from the *Bact. typhosum* Ty2 antigenic complex was not completely dissolved by treatment with 2*N* HCl for 2 hr. in a sealed tube at 100°. 0·008 g. quantities of the 'unextracted residue' were treated with 1 ml. 10*N* formic acid containing 3·6 % HCl and hydrolysed in sealed tubes at 100° for various lengths of time. At the end of the periods of hydrolysis the acid hydrolysates were exactly neutralized with NaOH and the amino-N determined by the Van Slyke method. The substance did not dissolve completely in the acid mixture even after 10 hr. hydrolysis. The results are summarized in Table 5.

Table 5. *Rate of increase of amino-N during acid hydrolysis of the 'unextracted residue' of the* Bact. typhosum *Ty2 antigenic complex*

Weight of sample mg.	Time hr.	Amino-N per ml. mg.	Total N per ml. mg.	Amino-N as % of total N
—	0	Substance insoluble	—	—
8·60	0·5	0·081	0·686	11·8
8·50	2	0·209	0·678	30·8
8·50	3	0·254	0·678	37·4
8·80	5	0·317	0·702	45·2
8·85	6	0·317	0·706	44·9
8·65	10	0·332	0·690	48·1

The results in Table 5 show that during acid hydrolysis the amino-N of the fraction of the antigenic complex (insoluble in acids and organic solvents)

increases to 48 % of the total N after 10 hr. hydrolysis, and that at this stage the liberation of amino-acids has reached a maximum. The insoluble nitrogenous component of the antigen contains tyrosine and arginine, it liberates 13·4 % of its total nitrogen as amino-groups on prolonged tryptic digestion, and 48 % of amino-N on acid hydrolysis and must therefore be regarded as a polypeptide.

The 86 % alcohol-soluble fraction of the antigenic complex

This fraction contains N 13·4 %, P (inorganic 0·2 %, organic 0·6 %), and has a small reducing power of 13 % (apparent glucose) which increases to 18 % on hydrolysis with 2*N* HCl in a sealed tube for 2 hr. at 100°. Of the total N, 3·0 % is in the form of amino-groups (Van Slyke). In aqueous solution the substance has a small dextrorotation, $[\alpha]^{20°}_{5461} + 7°$ ($c = 1$). The substance dissolves readily in cold water, leaving a trace of insoluble residue; a 0·4 % solution was subjected to various qualitative tests. Acid $KMnO_4$ solution was reduced in the cold. Positive biuret and ninhydrin reactions showed the presence of peptide linkages and amino-groups respectively, and that these were due to the presence of tyrosine residues was indicated by positive xanthoproteic and Millon's reactions. The presence of arginine was indicated by the Sakaguchi reaction, but the aldehyde test for tryptophan gave negative results. An immediate bulky precipitate was obtained, with phosphotungstic acid in 5 % H_2SO_4 and a precipitate on standing with neutral lead acetate, but no precipitate was obtained with 10 % trichloroacetic acid or tannic acid solution. A positive Molisch's reaction was obtained, but no furfuraldehyde was obtained on heating a sample with 12 % HCl, and Foulger's test for ketoses was also negative.

Whilst the alcohol-soluble fraction may be expected to contain any low-molecular substances liberated by acetic acid hydrolysis, e.g. sugars, amino-acids, etc., and the above quantitative and qualitative examinations would appear to confirm the presence of small amounts of these substances, there remains nevertheless about 10 % of N unaccounted for by amino-N. The presence of arginine and tyrosine and the precipitability of the fraction with phosphotungstic acid and neutral lead acetate are not inconsistent with the view that a water-soluble polypeptide may be present. Attempts to break down the substance by tryptic digestion and hydrolysis with mineral acids to produce free amino-acids have so far been unsuccessful.

Action of trypsin on alcohol-soluble fraction. 0·15 g. of the dried substance was dissolved in 15 ml. of a veronal-HCl buffer (*p*H 8·5), 0·003 g. trypsin (Fairchild Bros. and Foster, New York) added and the mixture incubated at 37° and *p*H 8·2–8·5; a further 0·003 g. trypsin was added after 2 days' digestion. Before addition of trypsin 7·5 % of the total N was present as amino-N (Van Slyke); this increased to 9·7 % after 2 days' digestion and 11·3 % after 5 days' digestion. It is evident, therefore, that the substance was not degraded to any significant extent by trypsin.

Acid hydrolysis of alcohol-soluble fraction. Acid hydrolysis of the alcohol-soluble fraction was investigated under the same conditions as those employed for the hydrolysis of the insoluble polypeptide fraction. 10 mg. samples of the dried solid were heated at 100° in sealed tubes with 1 ml. quantities of 10*N* formic acid containing 3·6 % HCl. After 2 hr. heating 12·4 % of the total N was liberated as amino-groups and 16·4 % after 7 hr., as compared with 45 % after 7 hr. in the case of the water-insoluble polypeptide. The action of conc. HCl [cf. Mitchell & Hamilton, 1929] on the alcohol-soluble fraction was also investigated. 10 mg. quantities of the substance were heated at 100° in sealed tubes with 1 ml. concentrated HCl; after 6 hr. 18·0 % of the total N was

liberated as amino-N and 18·3 % after 8 hr. heating. It appears unlikely that the alcohol-soluble component of the antigen is a polypeptide and the nature of this fraction remains obscure; investigation of this problem is being continued.

The lipid components of the antigenic complex

Taken as a whole the products of the acetic acid hydrolysis of *Bact. typhosum* Ty2 antigenic complex, which are soluble in organic solvents (light petroleum, ether, ethyl alcohol and acetone) constitute a total of only 2·7 % of the original antigen (Table 1). (In another experiment under similar conditions 4·3 % of substance soluble in these solvents was obtained.) Although other workers have isolated much larger amounts of lipid components of bacterial antigens [Morgan & Partridge, 1940; Soru & Combiesco, 1940] our *Bact. typhosum* Ty2 antigen does not appear to liberate a significant quantity of lipid components on acetic acid hydrolysis.

The light petroleum- and ether-soluble fractions described in Table 1 were combined by dissolving in an ether-alcohol mixture and N and P determinations made, giving N 0·6 %, P 0·5 % and N/P ratio 2·6. 0·15 g. of the lipid fraction was dissolved in 20 ml. ether and extracted with aqueous Na_2CO_3, when the bulk of the lipid material passed into the alkaline aqueous solution. This latter fraction was recovered by acidification and extraction with ether. The ethereal solution was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness, yielding 0·094 g. of a partially crystalline yellow-orange fatty substance. On analysis it gave N 1·2 %, P 1·0 %, N/P ratio 2·8. The material unextracted from ethereal solution by aqueous Na_2CO_3 was also recovered and found to be almost completely soluble in acetone. It appears, therefore, from a preliminary examination, that the lipid fraction consists predominantly of a mixture of fatty acids while a phospholipin may also be present in small amounts.

The action of trypsin on the tryptic digest antigen of Bact. typhosum *Ty2*

The antigens of Gram-negative organisms have generally been regarded as resistant to digestion by proteolytic enzymes [Boivin & Mesrobeanu, 1938, 1]. Morgan & Partridge [1940] have, however, been able to destroy the antigenicity of the *B. dysenteriae* (Shiga) antigen by prolonged treatment with relatively large quantities of trypsin. By tryptic digestion they degraded the complete antigen (a phospholipin-polysaccharide-polypeptide complex) into an antigenically inactive phospholipin-polysaccharide complex. It seemed unlikely that the *Bact. typhosum* Ty2 antigenic complex, which is isolated in an immunologically active and toxic condition by prolonged tryptic digestion of the bacterial cells, would be susceptible to attack by trypsin, but the further effect of relatively large quantities of the enzyme for a long period was investigated as follows. 0·206 g. of tryptic digest antigen was dissolved in 20 ml. veronal-HCl buffer (*p*H 8·5), and the solution treated with 0·008 g. trypsin (Fairchild Bros. and Foster, New York); the digest was incubated at 37° and *p*H 8·2–8·5. The course of the tryptic digestion was determined on 1 ml. samples of digest by the Van Slyke method for determination of amino-N. After 60 hr. digestion a further 0·03 g. trypsin was added and the experiment continued for 12 days in all. The results are summarized in Table 5.

Thus even when the antigen is submitted to prolonged attack by relatively large amounts of trypsin (approximately 20 times as much as that used in the tryptic digestion of the bacterial bodies) it undergoes an insignificant degradation

Table 5. *Rate of liberation of amino-N during tryptic digestion of* Bact. typhosum *Ty2 antigenic complex*

Time in hr.	Amino-N per ml. (mg.)	Total N per ml. (mg.)	Amino-N as % of total N
0	0·068	0·747	9·0
3	0·059	0·747	8·0
60	0·082	0·747	11·0
72	0·099	0·747	13·3
288	0·108	0·747	13·5

as indicated by liberation of only a further 4·5 % of the N as amino-groups. It is concluded that the tryptic digest antigen of *Bact. typhosum* Ty2 is stable towards further attack by this enzyme.

Discussion

Two main constituents of the antigenic complex of *Bact. typhosum* Ty2 have been isolated and investigated. These consist of a polysaccharide, which constitutes 50–60 % of the antigen and an acidic, water-insoluble polypeptide, constituting about 16 % of the complex. The polysaccharide, which has been obtained free from N and all but traces of P, gives on analysis C, 44·4; H, 7·1; acetyl, 3 %; and has been shown to consist of *d*-mannose, *d*-galactose and *d*-glucose units. An identical polysaccharide, $[\alpha]_D^{20°}+114°$, $[\alpha]_{5461}^{20°}+140°$, R.V. = 83 % apparent glucose, has been isolated directly from the dried bacterial cells by extraction with dilute acetic acid. Precipitation reactions with 'pure' O- and Vi-antisera have shown that the polysaccharide reacts specifically with O-antiserum but not with Vi-antiserum (in contrast to the antigenic complex from which it is derived), and is to be regarded as the O-specific hapten of *Bact. typhosum*. The polysaccharide probably represents, in a pure form, the soluble specific polysaccharide of *Bact. typhosum*, which White [1929] obtained, together with similar polysaccharides from other Salmonellas. Topley *et al.* [1937] also reported 'a highly dextrorotatory polysaccharide' on hydrolysis of virulent strains of *Bact. typhosum*. Earlier studies on crude bacterial polysaccharides have indicated that, as compared with the complete antigenic complex, they are relatively non-toxic and lacking in antigenic properties. Soru & Combiesco [1940] have isolated a polysaccharide on hydrolysis of the O-antigen of *Bact. typhosum*, with dilute acetic acid, which is probably identical with our polysaccharide though not yet pure. The polysaccharide of Soru & Combiesco is free from N, gives 72·4 % of reducing sugars on hydrolysis, is highly dextrorotatory ($[\alpha]+125°$) and contains 11·65 % of ash and 2·88 % of P (results, except that for P, calculated on an ash-free basis).

The polysaccharide hapten, which determines the specificity of the O-antigen of *Bact. typhosum*, has a parallel in the polysaccharide haptens of other organisms such as *B. dysenteriae* (Shiga), the specific polysaccharide of which, prepared by similar methods to those used in the present work, contains N (1·6 %), gives 97 % of reducing sugars on hydrolysis and is dextrorotatory ($[\alpha]_{5461}=+98°$). This polysaccharide is built up, in part, of *d*-galactose, *l*-rhamnose and *N*-acetylglucosamine units [Morgan, 1938; Jolles & Morgan, 1940].

The other well-characterized component of the *Bact. typhosum* Ty2 antigenic complex, the polypeptide-like component, probably plays a non-specific role in the complex antigen and is to be regarded as producing, in combination with the polysaccharide, a molecule of sufficient complexity to possess antigenic properties, the specific nature of which is dominated by the polysaccharide. The polypeptide component contains 8·2 % N and 2·4 % P; tyrosine and arginine

have been identified. The compound is fairly readily hydrolysed with 10*N* formic acid which contains 3·6 % HCl at 100° with liberation of 48 % of the N as free amino-groups (Van Slyke), but it is relatively very resistant to tryptic digestion. Soru & Combiesco [1940] obtained 21·7 % of an ether- and water-insoluble fraction from *Bact. typhosum* O-antigen. This fraction contained 6·7 % of N and only a trace of P. On hydrolysis with 2·5 % HCl the polypeptide-like fraction of Soru & Combiesco liberates a further ether-soluble constituent and virtually the whole of the N of the substance is liberated as amino-N. The latter observation appears to us surprising in view of the presence in the compound of arginine, and probably other amino-acids containing N other than as amino-groups. The corresponding polypeptide of *B. dysenteriae* (Shiga) contains 11·6 % N, tyrosine and arginine are present and again about 50 % of the total N is liberated as amino-N on acid hydrolysis [Morgan & Partridge, 1940].

Two other components of the antigenic complex have also been isolated but not yet investigated in any detail. Mixed lipid fractions amounting to 3–4 % were isolated in several experiments and appear to consist of a mixture of fatty acids together with a phospholipin. Soru & Combiesco [1940] obtained 15·8 % of an ether-soluble fraction from the *Bact. typhosum* O-antigen on hydrolysis with dilute acetic acid. The fraction was shown to consist of a mixture of fatty acids. Even larger amounts of lipid fractions have been isolated by Boivin & Mesrobeanu [1938, 2] on hydrolysis, with strong HCl, of the isolated O- and Vi-antigens of *Bact. typhosum*, which liberated 27·2 and 26·5 % of 'acides gras', respectively.

An alcohol-soluble nitrogenous fraction which comprises 10–20 % of the antigen has also been obtained. It contains 13·4 % N, the bulk of which is not present as free amino-groups and the percentage of these does not increase markedly on treatment with trypsin or on hydrolysis with acids. The nature of this component is still undetermined, and this problem is under investigation. The parts played by the alcohol-soluble component and the lipids in the complex antigen cannot yet be postulated but it is thought that they are of less fundamental importance than the polysaccharide and polypeptide components. Morgan & Partridge [1940] have shown that, although the *B. dysenteriae* (Shiga) antigen exists in the bacterial cell as a phospholipin-polysaccharide-polypeptide complex, the phospholipin component may be removed leaving a polysaccharide-polypeptide complex which is still specifically antigenic.

Preliminary work on an O-antigenic strain of *Bact. typhi-murium* has yielded similar results to those obtained with *Bact. typhosum* Ty 2, and led to the isolation of a polysaccharide fraction, an insoluble polypeptide-like component, a small amount of a lipid substance and a soluble nitrogenous component.

Summary

1. The *Bact. typhosum* Ty 2 antigen has been shown to consist of the following constituents, into which it may be dissociated on gentle hydrolysis: a polysaccharide component (50–60 %), an insoluble polypeptide (about 16 %), a soluble nitrogenous component (10–20 %) and a small lipid component (3–4 %).

2. The polysaccharide represents the O-specific hapten of the antigen; it has a reducing value on hydrolysis of 83 % in terms of glucose and is highly dextrorotatory ($[\alpha]_D^{20°} = +114°$). It appears to be free from ketose, pentose and uronic acid residues but yields *d*-galactose, *d*-mannose and *d*-glucose on hydrolysis. An identical polysaccharide has been prepared directly from the dried organisms by extraction with dilute acetic acid.

3. The polypeptide component is insoluble in water and soluble in dilute alkalis. It contains 8·2 % N, partly in the form of tyrosine and arginine units and about 50 % of the total N is liberated as amino-groups on acid hydrolysis.
4. The nature of the soluble nitrogenous component is, as yet, undetermined.
5. The antigen of *Bact. typhosum* Ty2 is resistant to tryptic digestion.

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