44. THE USE OF A SYNTHETIC MEDIUM IN THE ISOLATION OF THE SOMATIC ANTIGENS OF *BACT. TYPHI-MURIUM* AND *BACT. TYPHOSUM*

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SINCE the original description by Boivin & Mesrobian [1933] and by Raistrick & Topley [1934] of the isolation from organisms of the *Salmonella* group of non-protein chemical fractions possessing both toxicity and immunizing potency, other workers, and particularly Morgan [1937, 1, 2], have recorded work along similar lines. Three methods have been described by which such fractions can be isolated from the bacterial cell (1) by extraction with trichloroacetic acid [Boivin & Mesrobian], (2) by tryptic digestion [Raistrick & Topley], and (3) by extraction with diethyleneglycol. In another paper our colleague, Dr J. Walker, describes a fourth method which he has devised.

The work described in the present communication leaves little doubt that the fractions isolated by these different methods from any one organism are essentially the same. The work of the authors mentioned above indicates that the antigenic fractions consist essentially of a polysaccharide linked to a compound containing nitrogen and phosphorus, probably of a phospholipin nature. Their toxic and immunizing properties are closely similar, though there are minor differences [Henderson & Morgan, 1938] which indicate that the antigenic complex is probably affected, at any rate in its immunological properties, during the process of extraction. It is, however, extremely doubtful whether any one of the fractions yet prepared has been obtained in a state of chemical purity. The studies recorded in this paper, which are a continuation of those reported by Raistrick & Topley [1934], describe attempts to eliminate impurities derived from the starting material, or from the trypsin used for the digestion of the bacterial cells.

In all experiments referred to above, the large amounts of bacterial cells required for extraction have been obtained by cultivation on nutrient agar in Roux bottles, or in trays or Petri dishes, the growth being removed by scraping or washing. Since agar contains a large amount of polysaccharide it is clear that contamination of the starting material with this substance is likely to lead to contamination of a final fraction which itself consists largely of a complex polysaccharide. This difficulty has been encountered by other workers [Miles & Pirie, 1939].

This particular source of contamination can be avoided by growing the bacterial cells in a liquid medium, but such a highly complex medium as nutrient broth contains many constituents which might be carried through as contaminants to the final product. This difficulty can best be avoided by growing the bacterial cells in a simple synthetic medium.

We have found that commercial samples of trypsin, including that used in earlier experiments, contain large amounts of polysaccharide, while none are entirely free. The degree to which contamination from this source can be avoided is considered below.

The method described by Raistrick & Topley [1934] has been further improved by (a) removal of partially degraded cells by more efficient centrifuging of the tryptic digests and by filtration through Chamberland candles (L_2) and by (b) removal of low-molecular tryptic digestion products by dialysis in a collodion sac.

EXPERIMENTAL

(1) *Preparation of bacterial bodies*

The three methods employed in the preparation of the bacterial bodies which formed the starting material for our extractions were as follows:

(a) *Growth on nutrient agar in Roux bottles, and washed off.* Slight modifications of the method described by Raistrick & Topley [1934] were introduced. Batches of 90–100 bottles were put up at one time. Each bottle was inoculated with 3 ml. of an 18 hr. broth culture, incubated for 24 hr. at 37° and washed off with 7 ml. of 50% acetone in sterile tap water, glass beads being used to assist in dislodging the growth of organisms. The suspension of bacteria was transferred to wide-mouthed, glass-stoppered jars, and an equal volume of acetone was added. The jars were incubated for 3 days at 37° , and their contents stirred each day with sterile glass rods. On the 3rd and 4th days, each jar was tested for sterility, and kept in the incubator until proved sterile. The supernatant acetone was then removed, replaced by an equal volume of absolute alcohol, and a final sterility test was performed before the product was used for chemical fractionation. The bacterial bodies were then centrifuged down, washed twice with alcohol and twice with ether and finally dried *in vacuo* over conc. H_2SO_4 .

(b) *Growth on nutrient agar in metal trays; scraped off.* This method follows closely that described by Morgan [1937, 1]. Enamel trays $12 \times 10 \times 2$ in., each containing 500 ml. nutrient agar and covered with aluminium lids lined with filter paper, were inoculated with 50 drops (i.e. 1 ml.) of an 18 hr. broth culture, and the inoculum spread evenly over the surface by means of glass spreaders. After 24 hr. incubation at 37° , the growth was scraped off and suspended in sterile tap water in a wide-mouthed, glass-stoppered jar containing glass beads. An equal volume of acetone was added, and the subsequent treatment and sterility tests were the same as those used in the Roux bottle method. The supernatant acetone was removed by centrifuging and the bacterial bodies twice washed with alcohol at the centrifuge; two similar washings were then made with ether and the bacterial bodies dried *in vacuo* to a white powder.

(c) *Growth in synthetic medium.* Fildes *et al.* [1933] showed that although *Bact. typhosum* will not ordinarily grow in a medium without tryptophan, it can be trained to synthesize tryptophan when ammonia is the sole source of nitrogen. Recently, Gladstone [1937] studied the antigenic structure of *Bact. typhosum* grown in a synthetic medium which contained no tryptophan, and showed that in the presence of glucose the organism retains its Vi antigen, and is O-inagglutinable.

We have used the ammonia medium described by Gladstone, and, translating into quantities sufficient for the preparation of 60 l., the constituents are as follows:

KH_2PO_4	...	...	225 g.
$(NH_4)_2SO_4$	...	...	25 g.
NH_4Cl	...	...	25 g.

These salts are dissolved in 24,000 ml. of distilled water, and 1400 ml. *N* NaOH are added, the volume being made up to 30,000 ml. with distilled water. The reaction is adjusted to pH 7.6 by the addition of *N* NaOH, 18,250 ml. distilled water are added, and the medium is put up in 965 ml. quantities in 2 l. flasks and autoclaved at 115° for 20 min. To each flask the following solutions are added:

$M/6$ $MgSO_4 \cdot 7H_2O$	10 ml.	} Mixed and added in one operation.
$M/2$ Glucose	25 ml.	
M $NaHCO_3$	10 ml.	

The glucose was sterilized by steaming for 30 min., and the other two solutions by autoclaving at 115° for 20 min. It seems important that these solutions should be added not more than 24–48 hr. before the medium is to be used, as storing for a longer period leads to a reduction in the amount of growth. In practice they are added on the morning when inoculations are to be made.

Bact. typhi-murium grew readily in this medium, giving an opacity of 3000–4000 $\times 10^6$ organisms per ml. in 24 hr. We are indebted to Dr Gladstone for supplying us with a Vi strain of *Bact. typhosum* Ty₂ which had been trained to grow in a medium containing no added tryptophan. Both organisms remained fully virulent for mice when grown in the ammonia medium, and, apart from the loss of flagellar antigen, remained antigenically unaltered.

The usual procedure adopted for inoculating the flasks was as follows. The stock strains were kept on agar slopes in the ice chest, and subcultured about once a week. A generous loopful of culture was inoculated into a tube containing 10 ml. of ammonia medium and incubated for 24 hr. at 37°. About 5 ml. of this culture were put into a flask containing 250–500 ml. of ammonia medium, and incubated for 24 hr. at 37°. The following day 5 ml. of this culture were added to each litre of medium, and again incubated for 24 hr. at 37°. 5 ml. of 50% formalin in sterile tap water were then added to each flask (making a final concentration of 0.25%), and the flasks were stored at room temperature. After a further 24 hr., 0.5 ml. was removed from each and added to a tube containing 10 ml. of Hartley broth. These tubes were incubated for 3 days, examined daily for signs of growth, and plated on to agar and MacConkey medium on the 3rd day.

Recent experience has shown that satisfactory growth is obtained by seeding from the stock culture on the agar slope directly into the flask which is to be used for the mass inoculum, thus cutting out the preliminary growth in a tube of ammonia medium. The bacterial bodies were recovered from aqueous suspension in the culture medium by centrifuging in a De Laval Industrial Separator. The moist paste of organisms thus obtained was scraped from the bowl of the centrifuge, washed twice with alcohol, twice with ether and dried *in vacuo*.

(2) *Tryptic digestion of bacterial cells*

The technique of digestion of the dried bacterial bodies was based on the method of Raistrick & Topley [1934]. The organisms were submitted to digestion in batches of 10 g. The digestion mixture consisted of:

Dried bacterial bodies	...	...	...	...	...	10 g.
Sterile distilled water	...	...	...	...	...	2000 ml.
Trypsin (Fairchild Bros. and Foster, New York)	...	...	...	...	...	0.2 g.
Toluene	...	...	...	...	...	100 ml.

Half the trypsin was added at the beginning of digestion and the other half after 2.5 days. The digestion proceeded at 37°, pH 8.2–8.5 for 5 days. At the

outset sufficient alkali, usually about 4–5 ml. *N* NaOH, was added to bring the reaction to pH 8.2–8.5. The pH was determined approximately by means of a colorimetric method (B.D.H. comparator) and checked electrometrically, using a standard buffer and the "double hydrogen electrode". It was found that the pH tended to fall progressively during digestion and alkali was added when necessary to maintain the reaction at pH 8.2–8.5. Most of the alkali was required during the early rapid stage of digestion and the pH became fairly constant after 2–3 days. A typical digestion of *Bact. typhi-murium* bodies grown on synthetic medium required the addition of a total of about 18 ml. *N* NaOH. The course of the tryptic digestion has been followed by determination by the Van Slyke method of the amino-N liberated. Table 1 and Fig. 1 show

Table 1. *Rate of increase of amino-N during tryptic digestion of Bact. typhi-murium bodies*

Time hr.	Amino-N per ml. mg.	Total N per ml. mg.	Amino-N % of total N
0	—	0.609	—
1	0.055	0.609	9.0
3	0.073	0.609	12.0
8.5	0.102	0.609	16.8
23	0.147	0.609	24.2
30	0.151	0.609	24.8
48	0.165	0.609	27.1
60	0.172	0.609	28.2
72	0.196	0.609	32.2
77	0.205	0.609	33.6
82	0.209	0.609	34.3
96	0.209	0.609	34.3
102	0.215	0.609	35.3
120	0.212	0.609	34.8
456	0.223	0.609	36.6

Fig. 1.

that there is an initial rapid increase of amino-N during the first 24–36 hr. followed by a steady rise during the remainder of the digestion period. The addition of more trypsin at the 60 hr. stage produces a slight increment of amino-acid liberation. In this experiment the amino-acid content had reached 34.8% amino-N after 5 days and 36.6% amino-N after 19 days; thus there is little further enzymic hydrolysis after the first 5 days.

After 5 days' digestion the very opalescent creamy-grey coloured liquid was neutralized to pH 7.0 by addition of *N* HCl, evaporated *in vacuo* at low temperature to about half volume, and clarified to an almost water-clear solution by passage through a De Laval Industrial Separator. The insoluble residue was washed from the centrifuge with alcohol, and after further washing with alcohol and ether was dried *in vacuo* to a cream-coloured powder ("undigested residue") which constituted about 17 % of the bacterial bodies (*Bact. typhi-murium* grown in synthetic medium).

The cleared centrifugate was passed through Chamberland L₂ candles, giving a very slightly opalescent filtrate which was concentrated *in vacuo* to about 100 ml. volume and dialysed in collodion sacs at room temperature, first against running tap water for 18 hr., and then against frequent changes of distilled water for a further 24 hr. During dialysis there was a 100–200 % increase in volume and the solution became opalescent. The dialysed liquid was concentrated *in vacuo* to 25–50 ml. volume and centrifuged on an angle centrifuge at 4000 r.p.m. for several hours. The residue was dried down to a white powder ("dialysis precipitate"), and constituted 0.2 % of the bacterial bodies (*Bact. typhi-murium* grown in synthetic medium). At this stage the solution gives an increased opalescence on addition of acetic acid to 0.1–0.2 *N* concentration and deposits a precipitate on centrifuging ("acetic acid precipitate"). This procedure of removal of an "acetic acid precipitate" has been adopted in the preparation of certain of the antigenic fractions. In general, however, the cleared supernatant solution was treated with alcohol to 68 % concentration by weight to precipitate a fraction, known as "tryptic digest F 68", as a fine white powder. This was washed twice with alcohol, twice with ether and dried *in vacuo* over phosphorus pentoxide.

The possibility of trypsin or impurities therefrom being present in the final antigenic fraction has already been mentioned. Samples of trypsin were obtained from several sources and their properties determined. This investigation included determination of (a) tryptic activity, (b) polysaccharide content and (c) amount and nature of the fraction precipitated from aqueous solution by alcohol under the conditions used in the bacterial digestions.

As a result of these experiments it was found that Fairchild Bros. and Foster trypsin had approximately five times as great an activity as a sample of trypsin used in the preliminary experiments. The activities were compared by the formaldehyde method using caseinogen as substrate. Fairchild Bros. and Foster trypsin gave on analysis 9.9 % N and 10.6 % reducing sugars on hydrolysis in a sealed tube for 2 hr. at 100° with 2 *N* HCl (Hagedorn & Jensen's method) as compared with 5.6 % N and 44 % reducing sugars for the trypsin used in the early experiments. In a control experiment under conditions precisely similar to those used in the preparation of "tryptic digest F 68", 1.20 g. of this trypsin were extracted with 12 l. of water for 5 days at pH 8.2–8.5, and 37°, and the extract centrifuged, filtered through a Chamberland L₂ candle, dialysed and treated with alcohol to 68 % concentration by weight; this gave 0.07 g. "trypsin F 68", i.e. 5.8 % of the original trypsin; the fraction gave on analysis 6.7 % N, and 23 % reducing sugars on hydrolysis. (The sample of trypsin used in preliminary experiments gave, under similar conditions, 57 % "trypsin F 68", which gave on analysis 1.2 % N and 50 % reducing sugars on hydrolysis under the conditions stated above.) Assuming the yield of antigen to be 5 %, which is an average figure from our results, the "trypsin" content of the product cannot exceed 4 % of the total.

In some recent experiments an attempt has been made to avoid all possibility

of contamination with impurities from commercial samples of trypsin by digesting bacteria with purified trypsin prepared according to Northrop & Kunitz [1932] in their preparation of crystalline trypsin. So far, we have found it impossible to degrade the bacterial cells with the purified enzyme to the same extent as with the commercial samples, and this problem is being further investigated.

(3) *Preparation of antigenic fraction by Boivin's method*

The method depends on extraction of bacterial bodies with ice-cold trichloroacetic acid and is based on the original method of Boivin & Mesrobian [1933] and Boivin *et al.* [1933, 1, 2; 1934]. The dried bacterial bodies were dispersed in five times their weight of water by grinding in a mortar and rapid mechanical shaking for 30 min. The suspension was cooled to 0° and an equal volume of ice-cold 0.2*N* trichloroacetic acid added, the mixture being well shaken and kept at 0° for 3 hr. with occasional shaking. At the end of this period the very opalescent suspension was rapidly freed from the bulk of unextracted bacterial bodies by passage through a De Laval Industrial Separator, and the slightly opalescent centrifugate dialysed in collodion sacs at room temperature first against running tap water for 18 hr. and then against frequent changes of distilled water for a further 24 hr. After dialysis the trichloroacetic acid extract was again centrifuged through a De Laval Industrial Separator and the centrifugate passed through a Chamberland L₂ candle. The filtrates, which were usually still fairly opalescent, were concentrated *in vacuo* at low temperature and the specific bacterial substances precipitated by the addition of ethyl alcohol to 68% (by weight) concentration. The fraction thus precipitated was washed with alcohol and ether and dried *in vacuo* over phosphorus pentoxide to a white powder: "Boivin antigen F 68".

(4) *Preparation of antigenic fraction by extraction with ethyleneglycol or diethyleneglycol*

The method of extraction of bacterial bodies with ethyleneglycol and with diethyleneglycol used by Morgan [1937, 1, 2] for *B. dysenteriae* (Shiga) and Henderson & Morgan [1938] for strains of *Bact. typhosum* has been used in the preparation of antigenic fractions from *Bact. typhi-murium* and strains of *Bact. typhosum*. In our diethyleneglycol extractions we have used the distillate (B.P. 135–142° 10 mm.) of a commercial specimen of the solvent (B.D.H. "Technical"). The dried organisms (50 g.) were suspended in 500 ml. of the solvent at 37° by vigorous shaking in a stoppered bottle. A sample (0.5 ml.) of the suspension was removed, diluted with 4.5 ml. water and the pH determined electrometrically; this should be between 6.5 and 7.0. After 2 hr. at 37° the suspension was mechanically shaken for an hour and then kept at room temperature for 24 hr. During this period the suspension was frequently shaken by hand and finally mechanically shaken for an hour and kept for a further period of 24 hr. At the end of this time the bacterial suspension was freed from the bulk of suspended material by centrifuging in a De Laval Industrial Separator (or when smaller quantities of organisms were extracted the extract was clarified by centrifuging at 15,000 r.p.m. for 30 min.). The supernatant fluid was freed from any residual bacterial particles by filtration through a British Berkefeld filter candle followed by filtration through a Chamberland L₂ candle. The antigenic material was then obtained in aqueous solution free from the organic solvent by dialysis. During dialysis the slightly opalescent solution of the antigen in the organic solvent became strongly opalescent. At this stage the

antigen was isolated in a solid form by concentration of the solution *in vacuo* at low temperature and the addition of ethyl alcohol to 68% (by weight) concentration; the precipitate thus obtained was washed with alcohol and ether and dried *in vacuo* over phosphorus pentoxide.

The antigenic material soluble in the organic solvent is not always completely removed from the bacterial bodies by a single extraction. Where necessary, further extractions have been made in order to obtain a better yield; in these cases the number of extractions made is indicated in the relevant tables of results. These products have been labelled "ethyleneglycol or diethyleneglycol F 68".

The yield obtained by diethyleneglycol extraction of *Bact. typhosum* strain Ty₂ grown in synthetic medium (see Table 8) is considerably lower than that obtained by Henderson & Morgan [1938] and by Morgan (private communication), viz. 5% as a total of three extractions. In a series of subsidiary experiments the technique described above has been modified in attempts to improve the yield. These have included the use of only one bacterial filter, i.e. filtration of the diethyleneglycol extract through a Chamberland L₂ candle instead of filtration through a British Berkefeld filter candle followed by filtration through a Chamberland L₂ candle. This gave no significant increase in yield. When, however, cellophane membranes were used for dialysis instead of collodion sacs the yield was increased to 0.35%. Further experiments are proceeding with a view to determining the factors responsible for the differences between the yields we have obtained and those recorded by Morgan.

*Contamination of antigenic fractions with polysaccharide material
derived from agar or trypsin*

An appreciable amount of polysaccharide material can be removed from agar-grown organisms by aqueous washing. Thus 1.0 g. *Bact. typhi-murium* dried bodies, grown on agar medium in Roux bottles and washed off and killed with 50% acetone, was extracted three times with 100 ml. water and the extract centrifuged to remove bacterial bodies. The three extracts contained 0.122 g. (i.e. 12%), 0.039 g. (4%) and a trace respectively of solid material. The extracted material gave on analysis 33.2% reducing sugar on hydrolysis with 2N HCl for 2 hr. at 100° in a sealed tube (Hagedorn & Jensen method), 8.9% N and 8.9% non-volatile ash (the N and reducing sugar values are calculated on an ash-free basis). Aqueous washing of the bacterial cells, as a routine procedure to eliminate agar, was not considered satisfactory owing to the difficulties of eliminating the last traces of the soluble constituents by this method and the possibility of its removing part of the antigenic components of the cell.

Alcoholic or acetone fractionation of the antigenic fractions themselves was also tried, but it was found that these precipitating reagents behaved non-specifically in their precipitating actions on agar polysaccharides and the bacterial polysaccharides.

In the following experiment *Bact. typhi-murium* "tryptic digest F 68" from organisms grown on agar medium in Roux bottles was fractionated with alcohol and the fractions examined by determination of their specific optical rotations. Although this preparation contained about 14% of agar (see Table 5) there was no evidence of any laevorotatory compounds in any of the fractions obtained. (Agar yields a polysaccharide on alcoholic precipitation, $[\alpha]_{5461} = -35^\circ$, see Table 9.) 2.1 g. of the antigenic fraction were dissolved in 70 ml. water and alcohol gradually added until a slight precipitate was obtained. This precipitate was then removed by centrifuging, washed twice with absolute alcohol, twice

with ether and dried *in vacuo* over phosphorus pentoxide. More alcohol was then added to the alcoholic supernatant solution to produce further precipitation and the procedure repeated in 3–4 stages until the final alcoholic supernatant solution was evaporated to dryness. The largest of the fractions was then again refractionated as shown in Table 2; the results in this table are uncorrected for ash.

Table 2. *Alcoholic fractionation of Bact. typhi-murium "tryptic digest F 68" from organisms grown on agar medium in Roux bottles*

<i>Bact. typhi-murium</i> "Tryptic digest F 68" (Organisms grown on agar in Roux bottles)			
2.135 g. $\left\{ \begin{array}{l} [\alpha]_{5461} = +35^{\circ} \\ N = 2.2\% \\ \text{Agar} = \text{about } 14\% \\ (\text{see Table 5}) \end{array} \right.$			
45 %	54 %	70 %	Mother liquor residue
Fraction (1) $[\alpha]_{5461} = +31^{\circ}$ 0.535 g.	Fraction (2) $[\alpha]_{5461} = +38^{\circ}$ 1.286 g.	Fraction (3) $[\alpha]_{5461} = +34^{\circ}$ 0.264 g.	Fraction (4) — 0.018 g.
Redissolved in water (52 ml.) and refractionated			
Alcohol added to:			
56 %	70 %	Mother liquor residue	
Fraction (5) $[\alpha]_{5461} = +40.5^{\circ}$ $N = 1.8\%$ 0.938 g.	Fraction (6) $[\alpha]_{5461} = +39^{\circ}$ 0.270 g.	Fraction (7) — 0.006 g.	

Pirie [1936] describes methods for the detection and rough estimation of agar in biological materials. Use of these methods confirmed the presence of agar in antigenic fractions prepared from organisms grown on an agar medium in Roux bottles, but was of little value in determining its amount.

We have therefore adopted methods for roughly determining the extent of agar contamination of the dried bacterial bodies from various sources, depending on the galactose content of agar. It was found (*vide infra*) that bacterial bodies grown on a medium containing only mineral salts and glucose contained much less galactose than bodies grown on agar medium. These methods depend (a) on the oxidation of galactose to mucic acid, and (b) on the hydrolysis of the agar polysaccharides and precipitation of the galactose thus liberated as the methylphenylhydrazone.

(a) The oxidations were carried out according to Kent & Tollens [1885], who treated the galactose-containing substance on the boiling water bath with 10–12 times its weight of HNO_3 (sp. gr. 1.15), and evaporated to about $\frac{1}{3}$ – $\frac{1}{2}$ the original volume. After cooling, $\frac{1}{3}$ – $\frac{1}{2}$ of the original volume of cold water was added and the mixture well stirred to initiate crystallization. After 2–3 days' crystallization in the cold the mucic acid was collected on a small filter paper, washed with a few ml. of ice-cold water and dried at 100° . Under these conditions the oxidation of bacterial products was found to be complete in 1–2 hr. with 2 g. quantities of the substance. Mucic acid has also been obtained from the same

bacterial products by means of a shorter oxidation with conc. HNO_3 . About 2 g. of the substance were boiled with 5–10 ml. of conc. HNO_3 for about 10 min. The mucic acid which crystallized on cooling and vigorous stirring was filtered off on a small filter paper, washed with a few ml. of ice-cold water and dried to constant weight at 100° . The results obtained with various samples of dried bacterial bodies and antigenic fractions are given in Table 3, (i) by Kent & Tollens method and (ii) by the short oxidation method. Melting points of the products are quoted and refer to the mucic acid as obtained without recrystallization. The yields of galactose stated in the final column are calculated on the basis of a 78% yield of mucic acid from pure galactose. Kent & Tollens [1885] also obtained a yield of about 78% mucic acid from galactose.

Table 3. *Yield of mucic acid on nitric acid oxidation of dried bacterial bodies and antigenic fractions*

Substance	Wt. oxidized g.	Yield of mucic acid g.	M.P. of mucic acid	Galactose %
Galactose	(i) 2.0	1.558	217°	100
Agar (Bacto-agar)	(i) 2.0	0.286	208°	18.3
	(ii) 2.0	0.360	212°	23.1
B.D.H. trypsin	(i) 2.0	0.273	214°	17.6
	(ii) 1.7	0.225	211°	17.0
Fairchild Bros. and Foster trypsin	(i) 2.0	0	—	0
<i>Bact. typhosum</i> (R.R.) F 68 (organisms grown on agar medium in Roux bottles—digested with B.D.H. trypsin)	(i) 0.5	0.081	214°	20.8
<i>Bact. typhi-murium</i> dried bodies grown on synthetic medium	(i) 2.0	0	—	0
<i>Bact. typhosum</i> Ty ₂ dried bodies grown on synthetic medium	(i) 2.0	0	—	0
<i>Bact. typhi-murium</i> . Tryptic digest F 68. Organisms grown on synthetic medium digested with Fairchild Bros. and Foster trypsin	(i) 0.5	0	—	0

B.D.H. trypsin is the same sample as was used in the Raistrick & Topley [1934] work.

(b) Neuberg [1907] isolated galactose quantitatively, as the methylphenyl-hydrazone, from emulsin hydrolysates of raffinose. This method was subsequently used by Lüdtke [1929] and Neuberg & Schwietzer [1937] for the determination of galactose in agar and various polysaccharide fractions derived from it. Our procedure has been based on the methods of these authors; the bacterial bodies (2 g.) and antigenic fractions (0.5 g.) were hydrolysed on the boiling water bath for 3.5 hr. with 100 ml. $N \text{H}_2\text{SO}_4$.

Preliminary hydrolytic experiments with bacterial bodies showed that the acid hydrolysis was at a maximum after 3.5 hr. The hydrolysates were freed from any insoluble material by centrifuging and basic amino-acids and other protein degradation products were removed from the solutions by the addition of an excess (10 ml.) of 60% phosphotungstic acid in 5% H_2SO_4 . The mixture was kept overnight at 0° and the phosphotungstic acid precipitate removed by centrifuging and washed with 2% phosphotungstic acid in 5% H_2SO_4 . Excess

phosphotungstic acid and H_2SO_4 were removed by the addition of a very slight excess of hot saturated baryta to the acid hydrolysate, cooled in ice. The precipitated Ba salts were removed by centrifuging and washed with dilute baryta and the combined supernatant solution and washings made just acid by addition of $2N$ H_2SO_4 . The excess acid was removed by addition of a slight excess of pure BaCO_3 and the precipitates removed by filtration. The clear neutral and almost colourless filtrate was evaporated *in vacuo* at low temperature to 10 ml. volume. A few drops of dilute acetic acid were added during the evaporation to keep the solution just acid to litmus. 10 ml. absolute ethyl alcohol were then added and the mixture kept at 0° overnight; any precipitate which had separated out was removed by centrifuging. The filtrate was treated with 0.4 g. of methylphenylhydrazine (i.e. at least twice the calculated quantity) and 0.1 ml. 50 % aqueous acetic acid, kept at $35\text{--}38^\circ$ for 6–7 hr. with occasional gentle shaking and then in the ice chest for about 15 hr. At the end of this period the hydrazone obtained was collected on a tared Gooch crucible, washed with 10 ml. ice-cold ethyl alcohol and dried to constant weight *in vacuo* over phosphorus pentoxide. Where necessary the product was recrystallized from hot absolute alcohol until pure. Table 4 shows the results obtained for various bacterial products and a control experiment on pure galactose.

Table 4. *Determination of galactose in dried bacterial bodies and antigenic fractions*

Substance	Wt. g.	Yield of galactose methylphenyl- hydrazone g.	Nature of methylphenylhydrazone	Galactose %
Galactose	0.2239	0.3353	Colourless crystalline plates, M.P. 187°	95.0
<i>Bact. typhi-murium</i> dried bodies grown in synthetic medium	2.01	0.0234	Colourless micro-crystalline plates, M.P. 184°	0.74
<i>Bact. typhi-murium</i> dried bodies grown on agar medium in Roux bottles	1.809	0.0367	Colourless micro-crystalline plates, M.P. 185°	1.29
<i>Bact. typhosum</i> Ty ₂ dried bodies grown in synthetic medium	1.928	0.0195	Colourless micro-crystalline plates, M.P. 186°	0.64
<i>Bact. typhi-murium</i> "tryptic digest F 68" from organisms grown on agar medium in Roux bottles, digested with Fairchild trypsin	0.5739	0.0552	Colourless micro-crystalline plates, M.P. 183°	6.10
<i>Bact. typhi-murium</i> "tryptic digest F 68" from organisms grown in synthetic medium, digested with Fairchild trypsin	0.4908	0.0204	Colourless micro-crystalline plates, M.P. 184°	2.64
<i>Bact. typhosum</i> Ty ₂ "tryptic digest F 68" from organisms grown in synthetic medium, digested with Fairchild trypsin	0.4449	0.0080	Colourless micro-crystalline plates, M.P. 185°	1.14

Comparing Tables 3 and 4 it is found that the bacterial bodies from a synthetic medium and antigenic fractions prepared from them yield small though significant amounts of galactose on hydrolysis, which are detectable by the

sensitive methylphenylhydrazone method but cannot be detected by the cruder method of oxidative conversion of galactose into mucic acid.

If the assumption be made that the organisms synthesize the same amounts of galactose when grown on an agar medium as in the synthetic medium the above results quoted in Table 4 may be used to obtain a measure of the extent of agar contamination of bacterial bodies grown on agar media and of the antigenic fractions prepared from them.

Various workers have obtained figures of 25–30 % as the galactose content of agar (Neuberg & Schwietzer [1937], Lüdtke [1929] and Pirie [1936]) and we have obtained 23 % by the mucic acid method from unpurified agar. On the basis of a 25 % yield of galactose from agar, the following results are obtained for agar contamination of the bacterial bodies and antigenic fractions investigated.

Table 5

Substance	Organisms grown in synthetic medium: galactose %	Organisms grown on agar medium in Roux bottles: galactose %	Difference in galactose content %	Agar contamination %
<i>Bact. typhi-murium</i> dried bodies	0.74	1.29	0.55	2.2
<i>Bact. typhi-murium</i> "tryptic digest F 68"	2.64	6.10	3.46	13.8

In an attempt to reduce the agar contamination of the dried bacterial bodies grown on Roux bottles, two other methods of growth were investigated as described above. The dried bodies, prepared as already described from the three sources, have been submitted to chemical analysis and the results for N content and reducing sugar values, calculated on an ash-free basis, are given in Table 6.

Table 6. *Analysis of dried bacterial bodies*

	<i>Bact. typhi-murium</i>			<i>Bact. typhosum</i> R.R.	<i>Bact. typhosum</i> Ty ₂	
	Grown on agar medium in Roux bottles	Scraped from agar medium on trays	Grown in synthetic medium	Grown on agar medium in Roux bottles	Grown on agar medium in Roux bottles	Grown in synthetic medium
Yield	17.5 g. per 100 Roux bottles	73 g. per 100 trays	17.5–20 g. per 100 l.	15 g. per 100 Roux bottles	13 g. per 100 Roux bottles	13 g. per 100 l.
N %	13.7	14.8	13.4	13.4	13.0	13.4
Non-volatile ash %	8.2	3.8	6.5	8.6	9.5	7.4
Reducing sugars on hydrolysis for 2 hr. at 100° with 2 ml. 2N HCl in a sealed tube (Hagedorn & Jensen) %	14.1	13.9	16.4	14.5	14.6	14.9

Properties of the antigenic fractions

Various analytical results and the results of toxicity experiments in mice on antigenic fractions prepared from organisms grown by the methods 1 (a), (b) and (c) described above and fractionated according to the techniques described under (2), (3) and (4) are summarized in Tables 7 and 8.

Samples of antigens and bacterial bodies used in the various analytical determinations have been dried in small weighing tubes at 10–20 mm. and 61°,

with passage of a slow stream of dry air, for a standard time of 45 min. Just sufficient of the sample for the determination in question, e.g. 5–10 mg. for N determination, was dried in this way. The N analyses throughout the work have been made by the micro-Kjeldahl technique of Pregl. Ash determinations were made by ashing 5–10 mg. of substance in a platinum boat in a micro-muffle furnace. The reducing sugar determinations were carried out according to the method of Hagedorn & Jensen; 10 mg. quantities of substance were hydrolysed by heating on the boiling water bath with 2 ml. of 2*N* HCl for 2 hr. in a sealed tube; the hydrolysates were neutralized with 4*N* NaOH, diluted to 10 ml. and determinations made on 1 ml. samples. In the determinations of specific rotation about 100 mg. of substance were dissolved in 10 ml. water (a few micro-determinations were also made using 10 mg. of substance dissolved in 1 ml. water). The antigenic fractions dissolve to form slightly opalescent solutions and there is a tendency towards reversion to an insoluble form on drying which renders redispersion more difficult. Whenever it was impossible to determine the specific rotation on the solution at this stage, it was submitted to high speed centrifuging (usually 15,000 r.p.m. for 30 min.) until sufficiently clear for the determination to be made. Readings were made on the cleared supernatant solution in a 1 dm. tube using the mercury green line λ_{5461} Å. When there was separation of an appreciable quantity of insoluble residue on centrifuging, this was washed twice with alcohol, twice with ether, dried over phosphorus pentoxide and weighed. The weight of insoluble residue was deducted from the original weight of the sample in calculating the concentration of the rotation solution. The N contents, reducing sugar values and specific rotations are calculated on an ash-free basis.

The determinations of toxicity were made by the intraperitoneal injection into mice of 0.5 ml. of solutions containing falling amounts of the fraction under test. Each dose tested was injected into five mice, and the dose injected was halved in successive groups of mice, the range of dose employed in most tests being from 4 to 0.125 mg. with the various fractions obtained from *Bact. typhimurium*, and from 8 to 0.25 or 0.125 mg. with the fractions obtained from *Bact. typhosum*. For the method employed in calculating the average lethal dose, and its standard error, we are indebted to our colleague, Dr J. O. Irwin, who has recorded elsewhere [Irwin & Cheeseman, 1939] the statistical method itself and the results obtained in a series of parallel tests with some of our material. The standard errors have not been included in Tables 7 and 8, but it may be stated that, on the customary assumptions, the average lethal dose recorded must be regarded as subject to an experimental error of approximately +100 or –50 %, i.e. the true average lethal dose is unlikely to be more than twice, or less than half, the figure given.

Where many determinations have been made on similar preparations, as has been the case with the tryptic digest F 68 fractions from growth on Roux bottles, or in synthetic medium, the range given for the average lethal dose may be accepted with considerable confidence. Where only a single determination has been made the figure recorded should be treated with equal reserve. In a few instances in which an unusually wide range in A.L.D. is shown, as for instance, in the second tryptic digest fraction from the R.R. strain of *Bact. typhosum* (Table 8), a long period elapsed between successive tests on the same fraction. Since the higher A.L.D. values were obtained in the later tests it is possible that the toxicity of the fractions tend to decrease on storage, though in many samples we have found that the A.L.D. value remains relatively constant over periods of a year or more.

Table 7. *Properties of antigenic fractions prepared from Bact. typhi-murium*

Outline of method of preparation	Grown on agar medium and scraped off trays				Grown on agar medium in Roux bottles			
	"Tryptic digest F 68" V (a)		"Tryptic digest F 68" V (b)		"Tryptic digest F 68" I		"Boivin antigen F 68"	"Ethylene-glycol F 68"
	(Organisms acetone-killed) Dialysis in cellulose membrane		(Organisms acetone-killed) Dialysis in collodion membrane		(Organisms acetone-killed) VI		(organisms acetone-killed)	(organisms formalin-killed)
Acetic acid conc. <i>N</i>	—	—	—	—	0.1	—	—	—
Yield of fraction %	2.9	3.5	—	—	8.4	7.2	5.8	2.2
N %	4.3	2.8	3.4	3.0	2.4	2.6	3.3	4.3
Non-volatile ash %	8.2	7.4	8.3	6.6	7.6	7.1	9.6	8.2
Reducing sugar value %	39	52	43	56	54	49	47	39
[α] ₅₄₆₁	—	+90°	+65°	+91°	+38°	+43°	+34°	—
Average lethal dose for mice mg.	—	(approx.)	(approx.)	0.13	0.13-0.31	0.15-0.20	0.27-0.93	—
Outline of method of preparation	Grown in synthetic medium							
	"Tryptic digest F 68" VII	"Tryptic digest F 68" VIII	"Tryptic digest F 68" IX	"Tryptic digest F 68" XI	"Tryptic digest F 68" XII	"Tryptic digest F 68" XIII	"Boivin antigen F 68"	"Diethylene-glycol F 68" (1st and 2nd extractions)
	(approx.)	(approx.)	(approx.)	(approx.)	(approx.)	(approx.)	(approx.)	(approx.)
Acetic acid conc. <i>N</i>	0.2	0.3	0	0	0	0	—	—
Yield of fraction %	3.9	4.4	5.9	6.5	6.0	—	3.5	0.5
N %	3.0	5.4	6.1	4.1	6.0	4.1	2.5	2.4
Non-volatile ash %	6.1	9.6	12.5	8.2	9.7	7.4	9.0	9.7
Reducing sugar value %	51	48	47	51	47	51	48	51
[α] ₅₄₆₁	+75°	+83°	+83°	+80°	+80°	+77°	+47°	+78°
Average lethal dose for mice mg.	0.13-0.27	—	—	—	—	—	0.47	0.61

Table 8. *Properties of antigenic fractions prepared from strains of Bact. typhosum*

Outline of method of preparation	Strain R.R. grown on agar medium in Roux bottles				Strain Ty ₂ grown on agar medium in Roux bottles				
	"Tryptic digest F 68" II		"Tryptic digest F 68" I after treatment with 0.1 <i>N</i> HOAc in the cold		"Tryptic digest F 68" I		Strain Ty ₂ grown in synthetic medium		
	"Tryptic digest F 68" I	"Tryptic digest F 68" I	"Ethylene-glycol F 68"	"Ethylene-glycol F 68"	"Tryptic digest F 68" I	"Tryptic digest F 68" I	"Tryptic digest F 68" III	"Tryptic digest F 68" IV	"Boivin antigen F 68"
Acetic acid conc. <i>N</i>	0	0.1	—	—	1.0	0	0	0	—
Yield of fraction %	14	12	7.2	7.7	7.7	5.2	5.1	3.4	3.6
N %	5.2	5.0	5.7	4.2	4.2	7.7	6.8	7.0	4.2
Non-volatile ash %	11.5	11.1	21.3	9.1	9.1	12.0	12.5	12.2	9.1
Reducing sugar value %	37	42	39	46	46	30	35	32	36
[α] ₅₄₆₁	+38°	+42°	+45°	+81°	+81°	+112°	+93°	+107°	+90°
Average lethal dose for mice mg.	1.4	0.41-2.1	—	0.93	0.93	0.31	—	—	0.23

DISCUSSION

The three main methods of preparation of antigenic fractions which have been used in this work have yielded, from comparable starting materials, products which show remarkable similarities in chemical and immunological properties. They are all complex, non-dialysable substances containing *N* and giving rise to reducing sugars on hydrolysis. The fractions are all water-soluble, giving solutions which are optically active (dextrorotatory). The solutions exhibit the property, characteristic of their high molecular complexity, of

partial reversion to an insoluble form on drying and storage. Although the fractions at this stage cannot be regarded as chemical entities, the similarity of chemical composition and of toxicity in mice between the substances prepared by very different methods, from the uncontaminated starting material—bacterial bodies grown in a synthetic medium—lends support to the view that we are now dealing with a product which consists predominantly of the antigenic complex of the bacterium.

In view of the high percentage of reducing sugars obtained on hydrolysis of the antigenic fractions and the probability that a polysaccharide complex may play an important part in the immunological specificity of the antigen, it has been considered worth while to investigate systematically the problem of agar contamination of the starting materials and the antigenic fractions. The colour tests devised by Pirie, whilst confirming the presence of agar derivatives in the bacterial bodies grown on an agar medium and antigenic fractions prepared from them, were of limited value in assessing the amount of agar present. It has been possible, however, to obtain an accurate estimate of the extent of contamination of bacterial bodies and antigenic fractions by the determination of their galactose content, as compared with that of similar substances obtained from agar-free sources. The extent of agar contamination present in the dried bacterial bodies grown on an agar medium in Roux bottles has been determined by this means and appears to be of the order of 2% of the bacterial bodies. The results in Table 6 show that the organism grown in synthetic medium contained less N than bodies grown on agar and that the reducing sugars obtained on hydrolysis are correspondingly higher. This is probably associated with a change in the organisms occasioned by growth in a synthetic medium free from tryptophan. However, the immunological properties of the antigen obtained from bodies grown in this way do not appear to be materially affected.

Bact. typhi-murium antigenic fractions

Sample analyses of *Bact. typhi-murium* fractions prepared by three different methods from bacterial bodies grown both on agar media and in a synthetic medium are given in Table 7. In the case of the "tryptic digest F 68" from organisms grown in synthetic medium (antigenic solution untreated with acetic acid) four parallel preparations were made and the products analysed. The results are not strictly reproducible from batch to batch but the variations in the reducing sugar value (47–51%) and the specific optical rotation $[\alpha]_{5461} + 77-83^\circ$ are relatively small, although there is a greater variation in the N contents (4.1–6.1%). The same order of reproducibility is to be expected for the other fractions for which, in general, single analyses have been quoted.

Two fractions prepared from bacterial bodies, grown on nutrient agar in trays and scraped off, gave analytical results departing widely, particularly in N content, from all our other findings. The figures for these two samples are not included in Table 7. The cause of the discrepancy remains unknown.

The products prepared from organisms from an agar medium in Roux bottles by the three methods have N contents varying from 2.4 to 3.3%, reducing sugar values 47–54% and specific optical rotations, +34 to +43°, which present the same order of variation as the replicate batches quoted above. A similar argument holds for the fractions from organisms grown on agar medium and scraped off trays and for those fractions from organisms grown in the synthetic medium. The specific optical rotation (+47°) for the "Boivin antigen F 68" prepared from organisms grown in the synthetic medium differs widely from that obtained for the other fractions from this source; this may be

associated with differences of molecular complexity which would not affect the other analytical data.

The yields of the fractions prepared by the tryptic digestion method are higher than those prepared by the other methods.

Consideration of the average analytical figures for the products prepared by all three methods from organisms grown on the one hand on agar medium, and on the other on a synthetic medium, confirms the fact, established by the galactose estimations already described, that the antigenic fractions prepared from bacterial bodies grown in Roux bottles contain considerable amounts of "agar polysaccharides". The mean analytical figures may also be used to furnish a very rough indication of the extent of this contamination. Table 9 shows the average analytical figures for the antigenic fractions from the three main sources investigated, together with data for an agar polysaccharide fraction obtained by precipitating bacto-agar solution with alcohol to 68% concentration by weight, and calculated results for a hypothetical fraction consisting of 2 parts of antigenic fraction from organisms grown on the synthetic medium and 1 part "agar polysaccharide".

Table 9. *Mean analytical data of Bact. typhi-murium antigenic fractions*

	Method of growth of organism, from which fractions were prepared			"Agar poly- saccharide" fraction (b)	Hypothetical fraction 2 parts (a) + 1 part (b)
	Agar medium in Roux bottles	Agar medium in trays. Organisms scraped off	Growth in synthetic medium (a)		
Yield %	7.1	2.9	4.4	—	6.8
N %	2.8	3.4	4.2	0.5-0.6	2.9
Reducing sugar value %	50	48	50	50	50
$[\alpha]_{5461}$	+38°	+82°	+76°	-35°	+39°

Comparing our analytical figures with the values recorded in earlier papers, we find that the results are of the same order although there are certain differences, particularly in the N contents. Boivin's antigen from *Bact. typhi-murium* contained 1.83-2.20% N and gave 40% of reducing sugars on hydrolysis [Boivin & Mesrobian, 1934] as compared with our values, for the product obtained by the same method, of 2.5% N and 48% of reducing sugars on hydrolysis. Topley *et al.* [1937] obtained an antigen from *Bact. typhi-murium* containing 4.2-4.8% N; in our tryptic digestion work the N content of the purest fractions has varied from 4.1 to 6.1%. Raistrick & Topley [1934] obtained 23.1% of reducing sugars on hydrolysis of their crude antigen F 68 and 37.8% from F 68/68; our tryptic digest antigens from organisms grown in the synthetic medium yield 47-51% of reducing sugars on hydrolysis. These figures might appear to conflict with the view that the products of the earlier workers contained "agar" and other impurities from the culture media, but it must be remembered that efficient removal of bacterial debris by centrifuging and filtration, and elimination of low-molecular breakdown products of the tryptic digestion by dialysis rather than alcoholic fractionation, have also modified the analytical data of the fractions.

Bact. typhosum antigenic fractions

The mean analytical data for the *Bact. typhosum* fractions are summarized in Table 10.

Table 10. *Mean analytical data of Bact. typhosum antigenic fractions*

	Method of growth of organism, from which fractions were prepared, etc.		
	Strain R.R. Roux bottle grown	Strain Ty ₂ Roux bottle grown	Strain Ty ₂ Synthetic medium. Tryptic digest fractions
Yield %	11.1	7.7	4.8
N %	5.3	4.2	7.2
Reducing sugar value %	39	46	32
$[\alpha]_{5461}$	+42°	+81°	+104°

Comparison of these averages with the results in Table 8 shows that, firstly, parallel products from the organisms grown in the synthetic medium yield closely reproducible analytical data. So far as the products from a Roux bottle source are concerned, strain R.R. has yielded fractions by the three different methods of preparation which are closely similar, and it is again clear, as in the case of the *Bact. typhi-murium* fractions, that these three methods give rise to essentially the same polysaccharide products from the bacterial bodies. (In our hands the yields by extraction with glycols have always been lower than those by other methods.)

From a comparison of the analytical data for strain Ty₂ fractions prepared, on the one hand, from organisms grown on an agar medium in Roux bottles and, on the other hand, from organisms from a synthetic medium (Table 10) it is clear that the former contains an appreciable quantity of contaminating agar polysaccharides.

The N content of our "diethyleneglycol F 68" from strain Ty₂ grown in synthetic medium (4.3 %) is much higher than that quoted by Henderson & Morgan [1938] of 2.25–2.4 % N with a similar O + Vi strain. The same is true for the corresponding "Boivin antigen F 68"—4.2 % N as compared with 2.5 % obtained by Boivin. Topley *et al.* [1937] obtained a tryptic digest antigen from strain R.R. which contained 4.8–5.3 % N, and from the H 901 strain, 4.9–5.1 % N.

The analytical results in columns 1 and 2 of Table 8, and also those of *Bact. typhi-murium* fractions derived from the synthetic medium (Table 7) indicate that removal of an acetic acid-insoluble fraction of the antigen does not greatly alter its nature. It has already been pointed out that the antigenic fractions are readily susceptible to mutation to an insoluble form and it appears that treatment with acetic acid accelerates this, rather than produces a sharp separation of a chemically distinct fraction. Use of acetic acid is therefore not recommended at this stage of the preparation of the fractions.

Reference to Table 3 will show that an early crude antigenic fraction from strain R.R. organisms grown on agar medium in Roux bottles and digested with B.D.H. trypsin yielded mucic acid equivalent to 20.8 % of galactose. This is due to the presence in the antigenic fraction of galactose-containing polysaccharides from the commercial trypsin as well as of agar.

Boivin & Mesrobianu [1938] describe a method of separation of the O and Vi components of antigens from *Bact. typhosum* by means of precipitation of the Vi antigen with uranyl acetate. The precise chemical differences between these components have not yet been elucidated. We have preferred, therefore, at this stage to consider the antigenic complex from *Bact. typhosum*, strain Ty₂, as directly extracted by the methods described.

Comparison of antigenic fractions from Bact. typhi-murium and Bact. typhosum strain Ty₂ grown in synthetic medium

Both the antigenic fractions prepared from *Bact. typhi-murium* and *Bact. typhosum* strain Ty₂, organisms grown in synthetic medium and extracted by the three different methods, appear to belong to the same type of compound, i.e. a N-containing, non-dialysable water-soluble substance which yields reducing sugar on hydrolysis. They are both strongly dextrorotatory. The untreated antigenic fractions have no appreciable reducing power, and contain a negligible amount of amino-N. The fractions from the two organisms, though qualitatively similar, differ in their analytical data. Thus the fractions obtained from *Bact. typhosum* Ty₂ grown in synthetic medium all contain significantly greater quantities of N and have higher specific optical rotations than the corresponding fractions from *Bact. typhi-murium*. The reducing sugar values are however markedly lower.

SUMMARY

Methods of growth of bacterial bodies of *Bact. typhi-murium* and *Bact. typhosum* for the subsequent chemical extraction of antigenic fractions are described and have been critically examined. It has been found possible to obtain chemical fractions of these organisms free from contaminating substances, such as agar from the media, only when the organisms are grown in a liquid medium, preferably containing only inorganic salts and glucose.

Antigenic fractions have been prepared from these organisms by three methods: (a) a development of the tryptic digestion method of Raistrick & Topley; (b) Boivin's method of extraction with cold trichloroacetic acid and (c) extraction with organic solvents such as ethyleneglycol and diethyleneglycol [Morgan, 1937, 1], and the products have been compared by chemical analysis and the determination of toxicity in mice. The purest products obtained by these methods from organisms free from contaminants of the culture media show such a similarity of properties that it may be concluded that by any of these methods the essential antigenic complex of the bacteria may be obtained in a state approaching chemical homogeneity.

On the grounds of yield and suitability for use in extracting large quantities of bacterial bodies the tryptic digestion method and Boivin's method are recommended. Quantities of these products are being accumulated for further chemical fractionation.

A method for determining the galactose content of bacterial bodies and antigenic fractions has been elaborated and can be applied to the problem of the determination of the extent of agar contamination of bacterial fractions obtained from agar media.

These studies, and others in the same general field are, of necessity, extremely expensive. They involve the growth of relatively enormous masses of pathogenic bacteria, laborious and large-scale chemical manipulations, and the annual expenditure of many thousands of mice in the immunological assay of the various chemical fractions isolated. They have been rendered possible by grants received from many different sources: from the Leverhulme Trust, the Pilgrim Trust, Imperial Chemical Industries, Ltd. and from a fund raised by the late Sir Austen Chamberlain, to which many generous private donors have contributed. One of us (J. W.) has received a research grant from the Medical Research Council, two of us (G. G. F. and S. W. C.) have held Grocers' Company Scholarships for Medical Research, and Miss C. Begg, whom we wish to thank

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