45. A METHOD FOR THE ISOLATION OF TOXIC AND IMMUNIZING FRACTIONS FROM BACTERIA OF THE *SALMONELLA* GROUP

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Toxic and antigenically active fractions consisting of polysaccharides linked to a component containing nitrogen, phosphorus and fatty acids, but devoid of demonstrable protein have been isolated from bacteria of the coli-typhoid group by three different methods; by extraction with $N/4$ trichloroacetic acid [Boivin *et al.* 1933, 1, 2], by tryptic digestion [Raistrick & Topley, 1934] and by extraction with diethyleneglycol [Morgan, 1937; Henderson & Morgan, 1938]. The products obtained by these three methods from any one bacterial species correspond closely in their chemical, toxic and antigenic properties, though minor differences have been recorded in their antigenic behaviours. It would seem that they are very nearly identical although the methods used in their preparation have differed so markedly. Since, however, tryptic digestion has been used for the isolation of the bound polysaccharide from egg albumin [Neuberger, 1938] that method would seem to come under suspicion from the point of view of providing an entirely native product when applied to bacteria. The extremely acid nature of Boivin's solvent has been commented upon by that author himself [Boivin *et al.* 1938] and, as Morgan [1937] has emphasized, it seems improbable that the immunological properties of labile antigens will remain unaltered on exposure for any appreciable length of time to degrees of acidity and alkalinity which are far removed from neutrality. It was deemed desirable, therefore, to look for another technically simple method which should (a) be operative at a neutral reaction, and (b) introduce no substance incapable of subsequent ready elimination; these conditions are fulfilled by Morgan's [1937] method which yields toxic and immunizing products, but his procedure is lengthy and recent work [Henderson, 1939] would seem to indicate that an intact product is not obtained.

Experiments were therefore instituted along the following lines. It is well known that aqueous urea solutions have the power of peptizing proteins [cf. Spiro, 1900; Burk & Greenberg, 1930] and on dilution or on dialysing away the urea again the protein is frequently precipitated spontaneously [cf. e.g. Anson & Mirsky, 1929-30]. It was hoped, therefore, that by using urea solutions it would be possible to peptize the bacterial cells in such a way as to liberate the cell contents and then, by suitable means, either by dialysis or by the use of a specific precipitant, to render the protein insoluble and provide in this way a solution of the carbohydrate-containing protein-free antigen, which could subsequently be isolated in the free state. These expectations were, however, not realized since practically no dissolution of the cells could be demonstrated within the range of urea concentrations used [cf. also Fuller, 1938]. On the other hand, it was found that the solutions used had the power of extracting from the bacteria carbohydrate-containing, possibly protein-free, toxic substances which conferred a definite degree of antibacterial immunity on mice.

In the present work the organisms have been grown largely on the chemically defined medium described in the preceding paper by Drs Freeman, Challinor and Wilson, who discuss critically possible sources of contamination of bacterial bodies with agar, etc., since it is only after the elimination of all possible sources of contamination of the bacterial cells, either with components of the media upon which they are grown [cf. Sordelli & Mayer, 1931; Morgan, 1936] or with reagents used in their manipulation [cf. Delafield & Smith, 1939], that a reliable picture of bacterial synthetic activity will become available.

Preliminary experiments were carried out with *Bact. typhi-murium* and *Bact. typhosum* (strain R.R.) grown on nutrient agar and from these organisms toxic and immunizing fractions were isolated. Subsequent work was carried out with *Bact. typhi-murium* and *Bact. typhosum* (strain Ty₂) grown on the above-mentioned synthetic medium and only toxicity tests were carried out on the products obtained for reasons of economy both in mice and in time. No claim is made that the products were homogeneous and fractionation will have to await the accumulation of larger amounts than have hitherto been available. The yields obtained are of an order comparable with those obtainable by the method of Boivin & Mesrobian [1935] and rather less than those obtainable by that of Raistrick & Topley (see preceding paper); as far as can be seen at present the only significant differences between the present preparations and the products obtained by the other methods lie in the slightly higher nitrogen and lower carbohydrate contents of the urea extraction products where comparative figures are available. The likelihood of urea having entered into chemical combination with the bacterial product is not considered great and, since no significant amounts of protein can be present, some other reason must underlie the higher nitrogen values found for the present fractions. Prolongation of the urea extraction beyond 6–9 hr. to about 48 hr. appeared to cause a diminution in the toxicity of the product obtained and this observation may not be unconnected with that of Henderson [1939] that the product obtained by the somewhat lengthy diethyleneglycol extraction of *Bact. typhosum* had been reduced "to a position intermediate between that of the complete antigen of the intact cell and a true hapten". The possibility of a continuous slow enzymic destruction of the complete antigen showing up markedly in these cases of lengthy extraction should be borne in mind since it has been shown by Boivin & Mesrobian [1935] that the cells of *Bact. typhi-murium* and *Bact. enteritidis* contain an enzyme exerting precisely such an effect; they showed it to have an optimum pH of 7–8, to be inactivated by trichloroacetic acid and to be destroyed by heating to 100° or by trypsin at pH 8.5.

EXPERIMENTAL

Bacteria

These were supplied in the form of dried bodies by Professors Topley and Raistrick of the London School of Hygiene and Tropical Medicine. The methods of cultivation and killing were as follows:

Bact. typhi-murium: (A) 24-hr. cultures on nutrient agar; scraped off; acetone-killed. (S) 24-hr. cultures on the synthetic medium described in the preceding paper [Freeman *et al.* 1940]; formalin-killed.

Bact. typhosum (strain R.R.): (A) 24-hr. cultures on nutrient agar in Roux bottles; washed off; acetone-killed.

Bact. typhosum (strain Ty₂): (S) 24-hr. cultures on the synthetic medium; formalin-killed.

Extraction of bacteria with urea solutions

The following description is that of a typical experiment. 12.5 g. of bodies of *Bact. typhosum* (strain Ty₂), grown on the synthetic medium, were ground in a Griffiths tube and stirred for 9 hr. at 38° with 750 ml. of a 2.5 *M* solution of urea in sterile distilled water. The suspension was stored overnight in the ice chest in the presence of toluene and then clarified by passage through a De Laval centrifuge. The liquor retained by the centrifuge bowl (approximately 250 ml.) was displaced with 400 ml. of sterile distilled water, and the slimy residue left on the sides of the bowl was scraped out, treated with alcohol, washed with alcohol and ether and dried in a vacuum desiccator over conc. H₂SO₄; the dry residue amounted to 9.2 g.

The clear pale yellow liquor discharged from the centrifuge was dialysed in collodion sacs under a layer of toluene for 48 hr. against running tap water and for a like period against liberal quantities of distilled water with frequent changes. At the end of the dialysis the solution was paler and slightly opalescent; it was filtered through Chamberland L₂ candles and the filtrate, which retained a trace of opalescence, was evaporated to small bulk (77 g.) below 40° under 18 mm. pressure. During concentration opalescence increased; it increased still further on addition of the theoretical amount of 2 *N* acetic acid required to make the final concentration of acid *N*/10, but no significant sedimentation occurred when the acidified solution was centrifuged on an angle centrifuge at 4000 r.p.m. The supernatant (80 g.) was decanted and filtered alcohol (215 ml.) was added slowly with stirring until the concentration of alcohol reached 68% by weight. The resultant flocculent white precipitate (F 68) was collected at the centrifuge, washed with alcohol and ether and dried in a vacuum desiccator over conc. H₂SO₄; it amounted to 384 mg. (3.1% of the weight of dry bacteria used). The supernatant from F 68 was evaporated to small bulk (5–6 ml.) and treated with alcohol (ca. 100 ml.); the resultant precipitate (F "95") was collected and dried in the usual way and amounted to 62 mg. Total recovery of material, 9.65 g. (77% of weight of dry bacteria used).

The results of a number of extraction experiments are summarized in Table 1.

Properties of the various F 68 fractions

The products were all white or pale cream-coloured amorphous solids; they dispersed fairly readily in water to give slightly opalescent solutions which were in many cases sufficiently clear at 1% concentration to be polarizable through a depth of 5 cm. although accurate estimations of optical rotatory power were not attempted. All the F 68 fractions showed a positive xanthoproteic reaction and (approximately) 1% solutions gave a strong Molisch reaction, but in no case was a definite protein reaction shown with fresh ninhydrin solution although in the case of the typhoid preparations the reagent gave a faint pink colour on prolonged heating; the biuret reaction was positive though weak in all cases. Esbach's reagent and salicylsulphonic acid (used as 5% solution in 20% aqueous Na₂SO₄) gave barely perceptible turbidities of very similar order to those given by an 0.03% solution of gelatin which was used for comparison side by side with the tests on 1% antigen solutions; these tests, while they did not indicate the complete absence of intact protein, showed that no significant amounts could be present.

With uranium acetate only the *Bact. typhosum* preparations gave precipitates and these were soluble in citric acid solution [cf. Rouchdi, 1938; Boivin & Mesrobianu, 1938]. Estimates of the carbohydrate content of the various F 68

Table 1. *The extraction of bacteria with aqueous urea solutions, showing the results of toxicity tests*

Organism	<i>Bact. typhi-murium</i>			
	A (i)*	S (i)†	S (ii)	S (iii)
Preparation (F 68):				
Scale of exp. (g. bodies used)	7	12	10	12
Time of extraction at 38° (hr.)	6	48	8.5	9
Molar conc. of urea solution	2.5	2.5	2.5	6.5
% yield of F 68	1.7	5.7	0.5	2.5
Found for F 68:				
R.V. (%) after hydrolysis‡	—	26.6	—	35.3
N (%)‡	—	—	—	4.9
Ash (%)	—	—	—	9.7
Toxicity of F 68:				
Average lethal dose for mice (mg.)	0.23	1.23§	—	0.20

Organism	<i>Bact. typhosum</i>						
	Strain R.R.		Strain Ty ₂				
	A (ii)	A (iii)	S (iv)	S (v)	S (vi)	S (vii)	S (viii)
Preparation (F 68):							
Scale of exp. (g. bodies used)	20	10	2.5	2.5	12.5	24	30
Time of extraction at 38° (hr.)	45	9	9	9	9	9	9
Molar conc. of urea solution	2.5	2.5	2.5	6.5	2.5	2.5	2.5
% yield of F 68	3	14	3.0	3.5	3.1	3.6	2.9
Found for F 68:							
R.V. (%) after hydrolysis‡	35.6	21.8	—	20.4	23.1	23.1	29.2
N (%)‡	—	9.2	—	—	8.7	7.5	7.0
Ash (%)	—	11.6	—	—	8.3	9.9	12.6
Toxicity of F 68:							
Average lethal dose for mice (mg.)	>8§	<0.46	0.54	—	0.47§ 0.41	—	—

* A signifies "agar-grown" organism.

† S signifies organism grown on "synthetic medium".

‡ These values are *not* calculated on an ash-free basis.

§ A more resistant strain of mice used in these tests.

fractions were obtained by hydrolysing weighed amounts (10–40 mg.) with 2N HCl at 100° and then neutralizing carefully with 2N NaOH; after dilution to a definite volume, aliquot parts were used in the Hagedorn & Jensen [1923] procedure. The values found are recorded in Table 1 as "R.V. (%) after hydrolysis".

Toxicity of the F 68 fractions

Toxicity tests were carried out by Dr Joyce Wilson at the London School of Hygiene and Tropical Medicine. The fractions were tested by injecting mice intraperitoneally with falling doses from 8 to 0.125 mg. The method used in calculating the average lethal dose was that described by Irwin & Cheeseman [1939]. The results are appended to Table 1.

Immunizing potency of F 68 fractions

Tests of immunizing potency were performed by Dr Joyce Wilson at the London School of Hygiene and Tropical Medicine, using the technique of Raistrick & Topley [1934]; 30 mice were treated and 30 used as controls in each experiment; at the end of the immunization period, all the animals received the same lethal dose of living bacteria, and were observed over a period of 28 days. The results are shown in Table 2.

Table 2. *Showing the deaths out of 30 mice in each group in immunized and control groups*

Immunizing antigen	Infecting organism	Deaths	
		Immunized	Controls
A (i) F 68	<i>Bact. typhi-murium</i>	22*	26†
A (ii) F 68	<i>Bact. typhosum</i> (strain R.R.)	0	26

* Mean survival time, 16.2 days.

† Mean survival time, 11.6 days.

It will be noticed that the *Bact. typhosum* preparation A (ii) F 68 gave full protection to mice although it had lost much of its toxicity, while the highly toxic *Bact. typhi-murium* preparation A (i) F 68 was much less effective. This is quite compatible with the belief that mice should be immunized more readily against *Bact. typhosum* than against *Bact. typhi-murium*, since the latter is a natural mouse pathogen.

SUMMARY

Aqueous solutions of urea extract from bacteria of the *Salmonella* group substances which contain a considerable proportion of carbohydrate and at the most only traces of protein; these substances are highly toxic and confer active immunity on mice.

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