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# Preparation of Antigenic Material Inducing Leucopenia from *Eberthella typhosa* Cultured in a Synthetic Medium.

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Substances presumably free from whole protein which exhibit antigenic and toxic properties have been derived from cultures of the *Salmonella* group of organisms by Boivin and his associates,[1] and Topley and his coworkers.[2] The exact chemical nature of such agents as well as several of their immunologic aspects and effects on the animal body await further investigation. The present communication describes the occurrence of certain phenomena following the injection of mice and rabbits with a toxic, antigenic material prepared from cultures of *Eberthella typhosa* grown in a synthetic medium. The use of a medium containing ingredients which could all be removed by dialysis obviously should facilitate the separation of bacterial products from constituents of more complex media of unknown composition.

Adopting as a base the inorganic salts suggested by Burrows,[3] a synthetic medium was devised of the following composition: $(NH_4)_2SO_4$, 10 g; NaCl, 1 g; $K_2HPO_4$, 4 g; glucose, 7 g; acid hydrolyzed casein, 12 g; tryptophane, 0.2 g; cystine, 0.4 g; phenol red (0.2% solution), 10 cc, and distilled water, 2,000 cc. The reaction was adjusted to pH 7.4 and the mixture sterilized in the autoclave, except for the glucose, which was later added as a sterile 50% solution.

A recently isolated strain of *E. typhosa* possessing the characteristics of the smooth form was employed.

*Method.* 14 flasks each containing 2,000 cc of the solution were inoculated with 15 cc of a 12-hour culture of *E. typhosa* that had been cultivated in this same medium for 3 successive transfers. These flasks were incubated at 37°C. At 8-hour intervals, 10 cc of a 50% solution of glucose were added and sufficient NaOH to restore the pH to approximately 7.4. After 72 hours 10 g of sodium acetate

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1 Boivin, A., Mesrobeanu, I., and Mesrobeanu, L., *Compt. rend. Soc. d. Biol.*, 1933, **113**, 490.

2 Raistrick, H., and Topley, W. W. C., *Brit. J. Exp. Path.*, 1934, **15**, 113.

3 Burrows, W., *J. Inf. Dis.*, 1939, **64**, 145.

and 3 volumes of 95% alcohol were introduced into each flask which was then placed in the refrigerator for 48 hours. The supernatant fluid was decanted and the precipitate of cells and a grey, gummy material was recovered by centrifugation. These sediments from all the flasks were then combined by taking them up in 1,000 cc distilled water. The emulsion was shaken with glass beads for 2 hours at 37°C, placed in the refrigerator overnight and again shaken for 2 hours. After centrifugation, the solution was retained and the insoluble material resuspended in 300 cc of distilled water and the process repeated. The combined aqueous extracts, freed from particulate matter by centrifugation for ½ hour at 4,000 to 5,000 rpm, were placed in cellophane bags and dialyzed against running tap water at 10-14°C for 24 hours. Sodium acetate (0.5%) was added to the opalescent suspension: the reaction was made just acid to litmus with acetic acid and 3 volumes of alcohol added. After standing over night in the refrigerator, the precipitate was removed, resuspended in water and reprecipitated with 3 volumes of alcohol. This procedure was twice repeated. After the last precipitation the deposit was dissolved in 200 cc distilled water and shaken twice with 40 cc of chloroform and 8 cc of n-butyl alcohol according to the method of Sevag[4] to remove any protein present. Following the addition of 3 volumes of alcohol, the precipitate was washed twice with 70% alcohol and stirred with 100 cc of distilled water to give a light grey, colloidal suspension with a pH of 6.9 The yield was 0.8 g as determined by drying and weighing an aliquot of this solution.

Qualitative tests showed an absence of protein by the sulfosalicylic acid and $HNO_3$ tests. The biuret and ninhydrin tests were faintly positive. Millon and Hopkins-Cole tests were negative. A strongly positive Molisch reaction was obtained. After acid hydrolysis, treatment with phenylhydrazine yielded osazone crystals which were similar to those obtained with glucose. Elementary analyses gave: total N, 7%; amino N, 1%; total P, 5.6%. After hydrolysis with 1 N HCl for 1½ hours at 100°C, the product yielded 3.2% amino N and 13.4% reducing sugar (calculated as glucose). The ash was 24.4%.

*Antigenic properties:* With 3 typhoid antisera, the colloidal suspension induced a precipitate in a dilution of 1:200,000 (ring test 2 hours at 20°C). To 3 normal rabbits a total of 15 mg of this antigen in increasing doses were administered intravenously over a period of 3 weeks. Their sera exhibited agglutination titers of 1:1280 with a suspension of strain 0901 of *E. typhosa* and precipitin reactions with

[4] Sevag, M. G., *Biochem. Z.*, 1934, **273**, 419.

TABLE I.
Rabbit No. 5.

| Hours after injection | Body temperature | Total WBC count |
|---|---|---|
| 0 | 102.4°F | 11,750 |
| 0.15 mg antigen inj. intravenously | | |
| 2 | 106.2 | 2,600 |
| 4 | 106.6 | 2,350 |
| 8 | 104.8 | 11,000 |
| 24 | 104.0 | 21,150 |
| 32 | 103.6 | 14,000 |

the suspension of the antigen were positive in a dilution of 1:1,000,000 of the latter.

*Toxicity:* 4-5 mg of the preparation injected intraäbdominally into mice weighing 15-18 g killed 90% of the animals. Five rabbits of 1.5 to 2.5 kg body weight died within a few hours after intravenous injections of 0.4-0.5 mg. Intravenous administration of 0.1-0.25 mg was followed by marked leucopenia and fever in rabbits within 1 to 2 hours, subsequently attended by leucocytosis and a drop in temperature. Table I presents the results obtained in one of 10 rabbits so treated, all of which exhibited similar reactions.

Differential counts showed that the leucopenia resulted from the almost complete disappearance of the polymorphonuclear cells from the circulating blood. During the subsequent leucocytosis, these cells rose to a high level and many of them were found to be immature as indicated by the presence of single or double lobed nuclei. A number of years ago, a leucopenia in rabbits was described by Zinsser and Tsen[5] following large intravenous doses of suspensions of typhoid bacilli. While the present work was in progress, Smith[6] and Dennis and Senekjian[7] reported the isolation of leucocidal materials from cultures of *E. typhosa.* The material studied seems to differ from the leucocidal factor recently isolated by the latter authors in that it is precipitable from an acidified aqueous solution by the addition of alcohol. Twenty-four hours following the intracutaneous injection of 1 mg of the material into normal and immune rabbits, severe inflammatory reactions 2.5 cm in diameter appeared characterized by redness and marked edema.

The toxic properties of our substance appeared to be thermostabile, since boiling for ½ hour did not alter its capacity to kill rabbits or to induce a leucopenia and fever. A study of the relationship between its toxic and immunologic characteristics is in progress.

*Summary.* An antigenic substance has been isolated from cul-

5 Zinsser, H., and Tsen, E., *J. Immunol.*, 1916, **2**, 247.

6 Smith, E. V., *Am. J. Hyg.*, 1939, **29** (section B), 15.

7 Dennis, E. W., and Senekjian, H., *Am. J. Hygiene*, 1939, **30** (section B), 103.

tures of *E. typhosa* grown in a synthetic medium. Appropriate amounts kill mice and rabbits and smaller quantities induce a leucopenia and fever in rabbits.

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## Skin Sensitivity of Man to Bovine Plasma and Its Albumin and Globulin Fractions.

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The injection of whole bovine plasma in man was found to be associated with a considerable number of untoward reactions.[1] In an effort to determine the source of these reactions the whole plasma was fractionated into albumin and globulin portions. A study of skin sensitivity of man to these materials forms the basis of this paper.

*Method* From sterile bovine plasma, prepared as described elsewhere,[1] albumin and globulin fractions were separated by ammonium sulfate precipitation. The details of this procedure will appear in a subsequent publication.

One hundred and forty-six patients selected at random* were tested for skin sensitivity to bovine albumin solution, bovine globulin solution, and whole bovine plasma. Normal NaCl solution was used as a control. Five-hundredths cc of each of the above was injected intradermally and readings were taken after 20 to 30 minutes. No change or disappearance of the original wheal was considered negative, slight enlargement of the wheal with a small area of erythema 1+, a marked halo of erythema without pseudopodia 2+, pseudopodia and enlargement of the original wheal to twice original size

[1] Wangensteen, O. H., Hall, Harry, Kremen, A. J., and Stevens, B., in press.

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