A PRELIMINARY STUDY OF THE CHEMICAL NATURE OF THE TOXIC SUBSTANCES PRODUCED BY THE SALMONELLA GROUP OF ORGANISMS.

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The production of soluble toxic substances in broth cultures of these bacteria is well known. Berkefeld filtrates of such cultures are usually toxic for the rabbit upon intravenous injection. A review of the literature has been given by Ecker (1917, 1925) and by Branham (1925). Subsequent studies of these workers have shown that Berkefeld filtrates of synthetic medium (Braun and Cahn-Bonner, 1921) cultures yielded toxic substances inducing a similar reaction in the rabbit. Little is known concerning the nature of these substances beyond that they are relatively thermostabile; on dialysis of the broth culture filtrate the toxin is withheld within the collodium sac.

Considering the possibility of the production of these substances in a synthetic medium, their non-dialysable character and relative thermostability, a further study upon chemical lines seemed indicated.

Method.

The medium used was that of Braun and Cahn-Bonner (1921). It consisted of ammonium lactate 0·6 per cent., K_2HPO_4 0·2 per cent., and NaCl 0·5 per cent. The *p*H varied from 7 to 7·2. One to two litres of this medium were put up in 3 and 4 litre Florence flasks, sterilised at 15 lb. pressure for 15–20 minutes. Inasmuch as constant toxic filtrates were obtained from strains 180 and 185 (aertrycke)[1] these organisms were employed throughout the investigation. One loopful of a young culture was sown. Cultures of 3 to 5 days were passed through Berkefeld N candles. The filtrates were then injected into rabbits in doses of 3 c.c. Upon boiling of the cultures for 10 minutes prior to filtration similar symptoms of intoxication were observed on intravenous injection. As a precautionary measure, therefore, when handling large quantities, the culture flasks were placed in boiling water for 10 minutes. Over 100 litres have been prepared so far. Following filtration the filtrates were concentrated *in vacuo* at a temperature of 40°–50° C. to about one-tenth of their original volume. The concentrate was then subjected to dialysis in a gun-cotton sac until free from electrolytes, employing first tap-water and then distilled water. Dialysis was usually complete within 24–30 hours. Neutral lead acetate was subsequently added to the salt-free liquid and a precipitate obtained which

[1] These strains have been employed by Ecker (1917).

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could be centrifuged down and washed. Decomposition was effected by titurating the precipitate from 2 litres with 25 c.c. half-saturated $(NH_4)_2 SO_4$ in a mortar, leaving the mixture to stand over-night. Centrifugation and washing were repeated, the washings being added to the supernatant liquid. Only a trace of lead remained in the liquid and this was removed together with the excess of $(NH_4)_2 SO_4$ by a second dialysis. Some of the reacting material seems to go through the sac following this purification.

During the early part of the work attempts were made to effect concentration by adsorption. The following substances were used: Aluminium hydroxide (Willstätter and Kraut), kaolin, colloidal iron, precipitation of the phosphate from the medium and charcoal. Charcoal adsorbed or absorbed the toxic substances while the others failed. It was, however, impossible to recover the toxic substances from the charcoal by elution. Such methods were therefore temporarily abandoned. Precipitation of the phosphate from the Berkefeld filtrate was successfully accomplished by means of $CaCl_2$ and NH_3, the toxicity of the filtrate remaining unimpaired.

Experimental Results.

The typical symptoms of intoxication in the rabbit.

The animal exhibited, after a short incubation period of about 40 minutes, urination, hyperpnoea, prostration, diarrhoea and paresis of the posterior extremities. Recovery usually followed within 2 to 3 hours, a few animals succumbing with or without convulsions. Intravenous injections of as many as 6 and 9 c.c. of the sterile medium alone produced no ill effects in this animal.

Colour tests given by the Berkefeld culture filtrate.

The following colour tests were applied with the results indicated:

Buiret. Very faintly positive.
Millon. Negative.
Glyoxylic. Negative.
Diazo. Golden-yellow. Yellow in HCl, no secondary colour after reduction with zinc dust.
Molisch. Very faintly positive (also found by Branham (1927) in the case of *B. enteritidis*).

The following precipitants were also tried:

Saturated uranium acetate	No precipitate
Copper acetate	,, ,,
Phosphotungstic acid	,, ,,
Sodium tungstate	,, ,,
Mercuric acetate	,, ,,
Tannic acid	,, ,,
Hopkins' reagent	,, ,,
Saturated ammonium sulphate	,, ,,
Sulphosalycylic acid	,, ,,
Neutral lead acetate	White precipitate
Basic lead acetate	Heavy white precipitate
Silver nitrate	White precipitate

Colour tests, etc. given by the concentrated filtrate.

After concentration the filtrate gave a distinct Buiret, Molisch and Diazo-reaction, the other tests mentioned being still negative. In addition nothing was observed upon the addition of bromine water or sulphosalycylic acid. After dialysis the Buiret and Diazo-reaction were negative, the dialysate giving these reactions. Inasmuch as the toxic substances remained in the sac, the substances responsible for these tests were considered to be by-products of bacterial metabolism and not directly concerned in the toxic reactions. The concentrated and completely dialysed toxin was found to give certain colour reactions which are usually associated with the presence of carbohydrates. The following were observed:

Molisch	Strongly positive
Fehling solution	Very slight reduction. After hydrolysis with HCl, definite reduction
Phloroglucinol and HCl	Cherry red
Orcinol and HCl	Pink (faint)
Pyrogallol and H_2SO_4	Deep purplish black
Guaiacol and H_2SO_4	Green ring shading up into bluish purple

No measurable optical activity could be observed. Neutral lead acetate and $AgNO_3$ once more produced a precipitation. Alcohol precipitated a small quantity of material. Excess of baryta gives a white precipitate; acetone, however, failed to produce a precipitate.

The lead precipitate obtained after concentration and dialysis of the toxic filtrate was found to contain about 74 per cent. of lead, weighed as $PbSO_4$. The nitrogen content of different samples varied from 0·333 to 0·41 per cent. At the present stage it is left as an open question whether or not this quantity of nitrogen can be looked upon as representing an integral constituent of the molecule.

Four litres of strain 180 yielded 580 mg. of dried lead precipitate, whilst from five litres of strain 185, 430 mg. were obtained. The precipitate of the pooled filtrates of twelve litres of the two strains amounted to 910 mg. Taking as an average figure 90 mg. per litre, the toxic dose of 3 c.c. would represent 0·27 mg. of the lead precipitate, or 0·07 mg. of the lead-free material. Owing to the small quantities obtained, progress along chemical lines has been necessarily slow. On drying of the concentrated and dialysed filtrate over sulphuric acid *in vacuo* a gummy residue was obtained which did not readily re-dissolve. A similar material was observed from the decomposed lead precipitate. In both preparations the Molisch test was still positive.

Inasmuch as the $(NH_4)_2SO_4$ method of decomposition of the lead compound is far from quantitative, a method in which CO_2 and NH_3 is employed is being investigated and from which better results are hoped. On decomposing the lead compound with H_2S toxicity was always destroyed. H_2S blown through the toxic filtrate proper left it toxic after the H_2S had been removed by boiling.

Attempts to prepare an osazone from the HCl hydrolysed material have so far proved unsuccessful.

Skin reaction.

After decomposition of the lead precipitate by means of $(NH_4)_2 SO_4$ and dialysis the solution was found to give a reaction when intradermally injected into a rabbit. A marked skin reaction was obtained, the wheal being about 1·5 cm. in diameter within 24 hours. The centre was about 4 mm. in diameter, elevated and of whitish appearance, the surrounding area being hyperaemic. In about 4 days the central area had become necrotic while the hyperaemia subsided. This phase of the problem is being studied.

Table A. *Showing the reactions obtained in the rabbit before and after precipitation and decomposition of the toxic substances elaborated by strains* 180 *and* 185.

Reactions obtained with filtrates from boiled synthetic medium cultures

No.	Weight of rabbit in gm.	Strain	Age of culture days	Amount of filtrate injected in c.c.	Hour of injection	Result
1	1500	180	5	3	10.40	At 10.50 few faecal balls; 11.10 irritable, hyperpnoeic; 11.15 severe diarrhoea (continuous); 11.30 paresis of hind legs; heart very weak. Recovers
2	1980	185	6*	2	4.10	At 5.20 paresis of hind legs; marked defaecation; falls on side, hyperpnoeic and prostrated. Recovers
3	2650	180 and 185	3	3 (mixed filtrates)	10.35	At 11.0 marked defaecation; 11.5 hyperpnoeic; 11.40 marked diarrhoea; 11.45 paresis of hind legs; died at 12.10
9	1000	180 and 185	5*	3 (mixed filtrates)	10.00	At 10.25 urinates; 10.30 defaecates; 10.40 marked diarrhoea; 11.0 paresis of hind legs; falls on side; 11.27 heart weak, gasps and dies at 11.45

* Cultures stood respectively 24 and 32 days after boiling and prior to filtration.

Table B. *Reactions obtained in the rabbit with filtrates of strains* 180 *and* 185, *concentrated filtrates, concentrated and dialysed filtrates, and of the decomposed lead precipitate with* $(NH_4)_2SO_4$. *Age of culture,* 3 *days.*

No.	Weight in gm.	Amount injected in c.c.	Hour of injection	Result
26	1800	4	11·0	At 11.30 diarrhoea; 11.40 paresis of hind legs; severe diarrhoea; laboured respiration; falls on side; 12.20 prostrated and very sick. Recovers in the afternoon
27	1000	1·5 of concentrated (10 times)	4·10	At 4.30 defaecates; severe diarrhoea; extremely weak; 5.15 severe diarrhoea continuing; respiration laboured, appears to have cramps; dies during the night
28	1000	1·5 of concentrated and dialysed	11·15	At 11.30 urinates; soft faeces; 12.5 diarrhoea; prone; 12.10 paresis of hind legs; 12.20 severe diarrhoea; falls on side; very sick, dies within 48 hours
32	1900	2 $(NH_4)_2SO_4$ decomposed lead precipitate and dialysed	12·0	At 12.22 respiration rapid; 12.30 defaecates; 12.55 urinates; 1.0 prone; paresis of hind legs; 1.10 diarrhoea; 1.12 severe diarrhoea, cramps, general weakness. Recovers
34	1400	1 $(NH_4)_2SO_4$ decomposed lead precipitate and dialysed	2·40	At 3.0 defaecates; 3.20 diarrhoea; 3.45 severe diarrhoea; paresis of hind legs; 4.0 continuous severe diarrhoea; prostrated and irritable. Recovers

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Discussion and Summary.

Organisms of the Salmonella group (aertrycke) have been grown in a simple medium of known constitution. Growth in such a medium leads to the production of toxic substances. By filtration through Berkefeld N candles, concentration *in vacuo* and dialysis, the toxic substances were obtained in concentrated form free from diffusible materials. Addition of neutral lead acetate to this fluid apparently carries down all the toxic substances in the solution. The quantity of lead precipitate obtained was uniform for various samples. About 90 mg. of the lead compound were obtained per litre of culture filtrate, indicating that the experimental dose of 3 c.c. contained 0·07 mg. of lead-free material. Decomposition of this lead precipitate was effected by $(NH_4)_2SO_4$, the toxicity being unimpaired. In addition a skin reaction was observed in the rabbit following intradermal injections of 0·1 or 0·2 c.c. of the lead-free solution. In the preparations obtained we found small quantities of nitrogen, 0·3 to 0·4 per cent. Certain colour tests observed indicate the presence in these fractions of carbohydrate material. Owing to the delicacy of the colour reactions it is at present impossible to state whether the carbohydrate is merely a carrier of the toxic substance, a colloid impurity or an integral part of the molecule of the toxic compound. In the same way it is difficult to say what importance should be attached to the nitrogen figure. The recent work of Smith (1927) on an encapsulated form of *B. coli* and that of Heidelberger (1927) and co-workers upon the specific polysaccharides from the pneumococcus lend support to the view that the carbohydrate encountered in our toxic preparations may prove to be of significance[1]. The small amount of material causing the reaction would seem to indicate that the preparations are approaching purity. It has also been observed that the most refined material is not completely non-dialysable. Further investigations are proceeding upon chemical and immunological lines.

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[1] Since the preparation of this paper for publication a note has appeared by Tomcsik (1927) on a specific carbohydrate-like material in the capsules of the Colon Aerogenes group. As in the case of Smith and Heidelberger, the observations of Tomcsik relate to the encapsulating material of the organisms.