lation necrosis in which the dead tissues rarely undergo autolysis, except as a result of secondary infection." It is quite likely, therefore, that inhibition of autolysis is accomplished by the carbohydrate and phosphatide fractions of the tubercle bacilli.

It is also possible that the development of an increased susceptibility to the inhibitory action of the products of the tubercle bacillus which comes about as a result of infection is a defense mechanism since, as shown by Lurie,[14] tubercle bacilli usually die in caseous but grow in the softened areas. Furthermore, the former may undergo calcification and healing, whereas in the latter, the bacilli grow and from there disseminate to other sites.

Conclusions. The carbohydrate and phosphatide fractions of the tubercle bacillus (*M. tuberculosis*, H-37) exert a selective inhibitory action upon the endocellular enzyme, Cathepsin II, of tuberculous tissue. This phenomenon may help to explain the cytotoxic action of tuberculin *in vitro* and the failure of autolysis of caseous, tuberculous tissue.

[14] Lurie, M. B., *J. Exp. Med.*, 1933, **57**, 181.

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The Endotoxin of the Cholera Vibrio: Isolation and Properties*

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Though it is generally agreed that the cholera vibrio contains a potent endotoxin, the nature of the toxin is by no means clear. The isolation of a toxic protein has been reported by some workers such as Galeotti,[1] Sanarelli,[2] and Hahn and Hirsch.[3] More recently the trichloracetic acid extraction method of Boivin[4] has been applied to the cholera vibrio by Checcacci,[5] Raynal, Lieou, and Feissole,[6] Damboviceanu and Barber[7] and Gallut,[8] all of whom have reported successful extraction of a toxic fraction. It has been assumed by these workers that the active material is a polysaccharide-lipid complex, though in no instance is this conclusion supported by unequivocal evidence.

In the course of a study of active immunity to infection with *Vibrio choleræ*, we have had occasion to isolate the toxic principle from both Inaba and Ogawa types and partially purify it. Toxic solutions of the vibrio cell substance have been prepared in the following ways: (a) The cells are readily disintegrated in 4-5 hours by high speed grinding with sand, and the cellular debris is spun off, leaving a toxic opalescent supernate. (b) The cells may be dissolved in 6 M urea to give a similar toxic solution. (c) The cells may be digested with pepsin 3-5 days without inactivation of the toxicity, and the insoluble material removed by centrifugation to leave a toxic supernate. (d) The intact cells may be extracted in the cold with M/2 trichloracetic acid, all the toxicity going into solution. (e) Lyophilized cells may be extracted with methyl alcohol, ethyl alcohol, chloroform, or ethyl ether in a Soxhlet apparatus to give toxic

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago.

[1] Galeotti, G., *Centralbl. f. Bakt., I Abt. Orig.*, 1912, **67**, 225.

[2] Sanarelli, G., *Ann. Inst. Pasteur*, 1920, **34**, 370.

[3] Hahn, M., and Hirsch, J., *Centralbl. f. Bakt., I Abt. Orig.*, 1927, **104**, 211; *Klin. Woch.*, 1927, **6**, 312; *Z. f. Hyg. u. Infektionskr.*, 1929, **110**, 355.

[4] *Cf.* Boivin, A., and Mesrobeanu, L., *Rev. Immunol.*, 1935, **1**, 553.

[5] Checcacci, L., *Boll. Instituto Sieroterap. Milanese*, 1939, **18**, 391.

[6] Raynal, J., Lieou, Y. C., and Feissole, L., *Rev. Immunol.*, 1939, **5**, 317; *ibid.*, 1940, **6**, 132.

[7] Damboviceanu, A., and Barber, G., *C. R. Soc. Biol.*, 1940, **133**, 501.

[8] Gallut, J., *Ann. Inst. Pasteur*, 1943, **69**, 123.

extracts. Attempts to extract the toxicity from the intact cells with glycols have not been successful.

Of these the first method used was that of trichloracetic acid extraction. When the neutralized extract was dialyzed through cellophane to remove salt it was found that the activity appeared in the dialysate. The dialysis was rapid for toxicity was no longer detectable after 3-4 hours when carried out against running water. The toxicity may also be separated from the ground cells by dialysis though the process is slow, requiring 10 days in the refrigerator with daily changes of water. These observations are consistent with those of others, most recently Basu, Chaudhury, and Basu[9] and Banerjee,[10] who have reported that the toxicity is dialyzable.

The addition of 3-5 volumes of ethyl alcohol to the trichloracetic acid extract resulted in the appearance of a flocculent precipitate, which was shown to be polysaccharide, while the toxicity remained in solution. If polysaccharide is precipitated from concentrated extracts, the precipitate may contain some toxicity as an impurity but is readily purified.

When the filtrate from alcoholic precipitation was concentrated by evaporation under lamp and fan, a yellow oil separated out which contained most of the toxicity and had a mouse MLD of 0.1 cc. It appeared to be a mixture of alcohol and lipids. Similar material has been described by Raynal, Lieou, and Feissole.[6] Although studied somewhat further, preparations of this type did not appear to be promising because of the difficulty of separating the toxicity from trichloracetate.

The solubility of the activity in alcohol led to attempts to extract it directly from the vibrios in this and similar solvents. Extraction by stirring of hot or cold alcoholic suspension of the vibrios, or refluxing in alcohol, was found to be successful but inefficient. Extraction of the dry vibrios in a Soxhlet apparatus was highly satisfactory. With methyl alcohol, ethyl alcohol, and chloroform the extraction proceeds rapidly, the bulk of the activity being extracted in 1-2 hours. It is not clear whether removal is quantitative in 24 hours; at that time large doses, *ca.* 30 mg (60 billion vibrios), are required to kill mice by intraperitoneal inoculation and such deaths are difficult to interpret. On the basis of mouse assay, however, all the original toxicity may be accounted for in the extract. There is appreciable destruction of the activity after 6-8 hours in the boiling solvent and the entire toxicity may be removed intact only by fractional extraction. Extraction with ethyl ether is considerably slower, 7/8 of the activity being removed in 72 hours.

The extracted material was yellowish in color and appeared to be a mixture of lipids, and in the case of alcoholic extracts contained considerable quantities of inorganic salts. Similar preparations may be made by precipitation of ground vibrios with alcohol, etc. In general, however, extraction of dried vibrios with alcohol or chloroform was most satisfactory. The toxicity was precipitated from alcoholic solution by acetone, the solution becoming opalescent with the addition of 2 volumes and a white flocculent precipitate is formed with 10 volumes. Attempts to develop a fractional acetone precipitation were not successful; the activity precipitated partially at various acetone concentrations. The toxicity could be purified by successive acetone precipitation and re-solution in minimal quantities of hot absolute alcohol and obtained as a white lipid material. The yellow color of the crude extract was associated with inactive lipid. The substance so prepared was negative to the Molisch, Million, and biuret tests in alcoholic solution, alcohol-water solution, or aqueous suspension. Minimal values of nitrogen and phosphorus found on analysis were 5% and 0.7% respectively. With proper attention to inactivation in the extraction process, it could be consistently prepared with a mouse MLD of 30 μg. It was precipitated from alcoholic solution by the addition of water, the solution becoming opalescent when a concentration of 20-25% alcohol was reached, but did not flocculate unless the alcoholic solution contained as much as 1% of the material. It was inactive in suspension in alcohol-water or -saline, but the activity

[9] Basu, C., Chaudhury, A., and Basu, R., *Calcutta Med. J.*, 1940, **36**, 571.

[10] Banerjee, D. N., *J. Ind. Med. Assn.*, 1942, **11**, 95.

was restored by re-solution in alcohol. This material represented about 2% of the dry weight of the vibrios.

This material was obviously impure since the solubilities of the activity varied somewhat according to the method of preparation. For example, after more than 3 successive acetone precipitations of an alcoholic extract, the material became increasingly difficult to redissolve in alcohol. Alcohol solutions of chloroform extracted material gave a flocculent precipitate with acetone, but a second precipitation gave only an opalescence. Similarly, material prepared by ether extraction is less soluble in alcohol-water than that extracted with alcohol, precipitating in 50% alcohol. Such variability is, of course, characteristic of an impure lipid. In this connection it may be noted that very small yields of highly active material could be obtained from later fractions in fractional extraction; in some instances a mouse MLD of 2 μg has been observed. It seems probable also that some of the nitrogen represented contamination.

In general, however, it is clear that the endotoxin of the cholera vibrio is (a) resistant to peptic or tryptic digestion, (b) stable to acid, (c) unstable to alkali (N/10 NaOH at room temperature), (d) readily soluble in methyl and ethyl alcohols, chloroform, and ether but not in glycols, (e) readily dialyzable, and (f) is closely associated, possibly identical, with a phospholipid. These and additional studies will be reported in detail in a later paper.

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The Endotoxin of the Cholera Vibrio: Immunological Properties.*

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The toxicity of the cholera vibrio seems to play an important role in the pathology of Asiatic cholera in man and the immune response to this toxicity is of some interest. The isolation and purification of the vibrio endotoxin reported in the preceding paper[1] has allowed a more precise investigation of this matter than has hitherto been possible. Certain aspects of these studies are herein reported in preliminary form.

There appears to be considerable confusion regarding the antigenicity of the cholera toxin since many workers have designated hemolytic strains as "toxic" and have worked with antihemolysins. Since it is now established that the "true" cholera vibrio is non-hemolytic,[2] such studies would seem to be irrelevant. Some workers, including Pottevin,[3] Horowitz,[4] Bail,[5] Hahn and Hirsch,[6] and Andu and van Niekerk[7] have reported the preparation of antisera which were protective *in vivo* against the lethal action of toxic extracts of the vibrios. It is generally admitted, however, that the

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago.

1 Burrows, W., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 306.

2 See, for example, the earlier studies of van Loghem, J. J., *Centralbl. f. Bakt., I Abt. Orig.*, 1926, **100**, 19; also Gardner, A. D., and Venkatraman, K. V., *J. Hyg.*, 1935, **35**, 262.

3 Pottevin, H., *Bull. Soc. Path. Exot.*, 1913, **6**, 409.

4 Horowitz, L., *Z. f. Immunitätsf.*, 1913, **19**, 44.

5 Bail, O., *Z. f. Immunitätsf.*, 1916, **25**, 248; *ibid.*, 1917, **26**, 97.

6 Hahn, M., and Hirsch, J., *Centralbl. f. Bakt., I Abt. Orig.*, 1927, **104**, 211; *Z. f. Hyg. u. Infektionskr.*, 1929, **110**, 355.

7 Andu, A. B., and van Niekerk, J., *Centralbl. f. Bakt., I Abt. Orig.*, 1929, **112**, 519.