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.

14792 P

The Endotoxin of the Cholera Vibrio: Immunological Properties.*

WILLIAM BURROWS, ADALINE N. MATHER, SYLVIA M. WAGNER, AND VIRGINIA G. MCGANN.

From the Department of Bacteriology and Parasitology, the University of Chicago.

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.

antitoxic properties of antisera are only feeble at best. For example, Bail[8] attempted to account for the unaltered toxicity of serum-treated vibrios by the demonstration of free complement-fixing substances in immune guinea pigs infected with vibrios, these substances presumably representing uncombined toxin. According to Eisler and Kovacs[9] the toxin is not antigenic in that it is not specifically precipitated by antiserum, but adsorbed on the flocculating antigen-antibody complex.

In our experiments various preparations of endotoxin have been studied. The immunological activity of endotoxin prepared by preliminary extraction with alcohol and 3 subsequent precipitations with chilled acetone was indicated by skin reactions in immune rabbits and by specific precipitation and complement fixation with rabbit immune sera. The intradermal inoculation of 100 to 300 μg of endotoxin in 0.1 ml 25% alcoholic solution gave a skin reaction of the delayed type, appearing in 24 to 36 hours and fading appreciably in 48 hours, in immune rabbits but not in normal rabbits. An immediate toxic reaction, attributable only in small part to the alcohol and characterized by a circumscribed erythema followed by superficial necrosis, appeared in 1 to 3 hours and persisted for several days. It was not appreciably less in immune animals.

Specific precipitation could be demonstrated by the ring test and by a precipitation test of the Kahn type. In the former the antigen was used in solution in 25% alcohol and layered over the serum in the usual way; 25% alcohol gave a very slight clouding at the interface with both normal and immune sera which was not confusing. The precipitate appeared in 15 to 30 minutes at 37°C and disappeared almost entirely after 2 hours. Precipitation with H-O and O antisera occurred with antigen diluted to 1:5,000 to 1:10,000 as a rule though positive reactions were occasionally observed in dilutions as high as 1:400,000. With undiluted serum nonspecific reactions, *i.e.*, precipitation of the antigen with normal serum, were common and usually occurred to about ¼ the titer observed with immune sera. These could be reduced to 1:50 or less by 1:5 dilution of the serum; similar dilution of immune sera reduced the precipitin titer to 1:500 to 1:1,000.

Solutions of endotoxin preparations in absolute alcohol were used as antigens in the Kahn type of precipitation test. The antigen was found to be dispersed (cloudy but not granular) when mixed in the proportion of 0.01 ml antigen to 0.15 ml saline if the concentration of the endotoxin preparation was reduced to 0.72 mg per ml. This antigen was used both plain and cholestrinized (treated with 2 mg cholesterol per ml) and set up in 0.01 ml amounts with 0.15 ml of 1:5 and higher dilutions of inactivated serum. The cholestrinized antigen was found to be superior as indicated by more complete precipitation and often precipitation at higher serum dilutions. Readings were made immediately after shaking and were somewhat more clear-cut after standing 15 minutes. No precipitation was observed with normal rabbit serum. H-O and O antibacterial sera gave 3+ (flocculent precipitate without clear supernate) reactions in dilutions as high as 1:50 and 1:60, and a single antitoxic serum (see below) gave 4+ in 1:60 dilution.

The absolute alcoholic antigens were highly anticomplementary and the anticomplementary unit, approximately 0.35 mg, was unaffected by dilution in distilled water, 25% alcohol, physiological salt solution, and various concentrations of normal serum. Antigenicity disappeared almost entirely in distilled water dilution, the antigenic unit was approximately equal to the anticomplementary unit in 20% alcohol and serum dilution, and was about 4 times as large, *i.e.*, 0.09 mg, in saline dilution. The antigen was, therefore, used in saline dilution. Heating the diluted antigen at 56°C for 60 minutes did not affect its anticomplementary activity but reduced antigenicity by about half. Treatment with cholesterol increased both anticomplementary activity and antigenic activity, the former to 0.035 mg and the latter to 0.014 mg. In spite of the unfavorable alteration in the anticomplementary unit-antigenic unit ratio the cholestrinized antigen was more satisfactory

[8] Bail, O., *Z. f. Immunitätsf.*, 1916, **24**, 396.

[9] Eisler, M., and Kovacs, N., *Wien Klin. Woch.*, 1926, **39**, 469.

in the complement fixation reaction. In no instance did fixation occur with this antigen in the presence of normal rabbit serum. Only partial fixation, rarely 3+ and usually 2+, occurred with H-O, O and antitoxic sera, and in general 2+ fixation was observed in serum dilutions as high as 1:50. Antitoxic sera did not fix complement in higher dilution nor more completely than the antibacterial sera.

These observations appear to indicate at least a haptene-like function of the purified endotoxin preparations, though thus far the *in vitro* antigen-antibody reactions are relatively unsatisfactory for routine use.

In view of the low molecular weight indicated by its ready dialysis, it was not anticipated that endotoxin would stimulate antibody formation. Nevertheless, a series of rabbit immunizations was attempted with various types of preparations, including: (1) absolute alcohol solution of crude alcoholic extract; (2) suspension in 20% alcohol of alcoholic extract purified by 3 successive precipitations with chilled acetone (mouse MLD of final preparations was 35 to 40 μg); (3) preparations of dialysate of ground vibrios, precipitated after grinding with minimal amounts of trichloracetic acid, and dialyzed against daily changes of distilled water in the refrigerator for 14 days, (a) dialysate concentrated to contain 1 mouse MLD per ml, (b) dialysate evaporated to dryness, taken up in absolute alcohol, filtered and diluted to contain 20% alcohol (mouse MLD of final preparation 25 μg).

In the first experiments with alcoholic solutions only small amounts of material could be given because of toxicity; the lethal dose of endotoxin for the rabbit has not been determined accurately but appears to be possibly double that for the mouse, disregarding differences in body weight. A representative animal receiving a total of 42 μg of endotoxin in 3 intraperitoneal and 2 intravenous doses in a period of 14 days showed no agglutinin titer (<1:100) but in 0.1 ml amounts the serum protected mice against 100,000 MLD of mucin-suspended vibrios.† An additional intravenous inoculation of 3 mg was tolerated but demonstrable agglutinins did not appear. A second dose of 3 mg resulted in an agglutinin titer of 1:500 but there was no increase in protective titer. These, and similar results with other animals, were taken to suggest the independence of agglutinin and protective antibody.

As pointed out in the preceding paper,[1] suspensions of the alcoholic endotoxin in 20% alcohol in water or physiological saline were not toxic and larger amounts could be given. A number of preparations of acetone-precipitated material were used and the following experiment is representative. Following the intraperitoneal and intravenous inoculation of a total of 8.0 mg in 5 doses, the agglutinin titer was 1:1,000 and the serum protected passively immunized mice against 100,000 MLD of mucin-vibrios. Two additional intravenous inoculations of 1.6 mg each did not raise the agglutinin titer; in fact it fell to 1:500, but the protective titer rose to between 1,000,000 and 10,000,000. One animal in the series showed a very high agglutinin titer, 1:20,000, but similar titer of protective antibody.

The antisera to these alcoholic antigens have not been examined systematically for precipitin and complement-fixing antibody titer, but the few which were tested gave positive reactions.

In the experiments with dialysate it is to be emphasized that the barrier of dialysis through cellophane was interposed between the intact vibrio and the final preparation; consequently, no matter what the impurities in such preparations, large molecules that do not dialyze are not present. The animals immunized with the 2 types of dialysate preparations indicated above received a total of 7.5 to 8.5 mg in 5 or 6 doses, only the first of which was intraperitoneal. In all instances the protective titer was at least 100,000 and the agglutinin response marked, with all sera showing titers of 1:50,000 or more. As measured by agglutinin response, these antigens appear to be markedly superior to whole vibrios for a concentrated course of hyperimmunization with a total of 50 to 100 mg (100,000 to 200,000 million vibrios) has been

† Other aspects of this investigation, including detailed studies on protective antibody, are as yet unpublished.

found necessary to produce high agglutinin titers.‡

Mice could be actively immunized by the inoculation of alcohol-saline suspension of alcoholic toxin. In a typical experiment a total of 234 μg given in 3 intraperitoneal inoculations 4 days apart protected against 10,000 MLD of mucin-vibrios given 6 days after the last inoculation. In comparison with this, approximately the same degree of protection is afforded by the inoculation of not less than 1 mg of whole vibrios in heat-killed vaccine, and often a larger amount has been required.

On the other hand, it has not been possible to demonstrate an *in vitro* neutralization of the activity of endotoxin preparations by either antibacterial or anti-endotoxic sera known to be protective when tested against mucin-vibrios in the mouse. Thus, the toxicity of concentrated alcoholic solutions containing 1000 MLD per ml was not impaired by mixture with 2 volumes of antiserum having a protective titer of 100,000, the mixture being inoculated in 0.05 ml amounts either immediately or after incubation for as long as 6 hours. Furthermore, we have been unable to immunize mice, either actively or passively, against the lethal effect of intraperitoneal inoculation of the purified endotoxin. These titrations have all been carried out with dilution in powers of 10; it is possible that the more sensitive method of proportionate deaths would indicate some protection, but we have not used it because of its limited utility.

‡ Details of immunization procedure and agglutinin response will be reported elsewhere in a study on the antigenic structure of the cholera and related vibrios.

14793 P

The Endotoxin of the Cholera Vibrio: Action on Living Semipermeable Membranes.*

WILLIAM BURROWS, SYLVIA M. WAGNER, AND ADALINE N. MATHER.
From the Department of Bacteriology and Parasitology, the University of Chicago.

The profuse diarrhea, a prominent feature of Asiatic cholera in man, is usually attributed to the action of the endotoxin of the vibrios; whether the pathology of organs such as the kidney is produced by absorbed toxin or is a consequence of the marked dehydration and hypochloremia is not clear. Though in general the pharmacological activity of the bacterial endotoxins is not characteristic, some workers have reported that intravenous inoculation of experimental animals with toxic extracts produces a symptom complex analogous to that of the early stages of human cholera. Hahn and Hirsch,[1] for example, reported that a profuse diarrhea with a loss of 10-17% of the body weight of fluids is produced in young rabbits and Ghosh[2] has made similar observations. Pham[3] has produced a diarrhea accompanied by marked albuminuria leading to emaciation and death in guinea pigs and rabbits; postmortem examination showed hemorrhagic infiltration of the terminal portion of the small intestine, congestion of Peyer's patches and desquamation of the mucosa together with some pathology of the kidney. Less characteristic findings have been reported by Sanarelli[4] and by Basu, Chaudhury, Basu.[5]

* 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 Hahn, M., and Hirsch, J., *Klin. Woch.*, 1928, **7**, 2483.

2 Ghosh, H., *C. R. Soc. Biol.*, 1933, **112**, 1176.

3 Pham, H. C., *C. R. Soc. Biol.*, 1935, **119**, 78.

4 Sanarelli, G., *Ann. Inst. Pasteur*, 1920, **34**, 370.

5 Basu, C., Chaudhury, A., and Basu, R., *Calcutta Med. J.*, 1940, **37**, 571.