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.

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

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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.

In a few instances the effect of toxic extracts on isolated tissues, heart and intestine, has been studied. Perfusion of the heart with toxic extracts is reported to produce paralysis[6] and peristalsis of the intestine is said to be stimulated by small doses of toxin and inhibited by large doses.[1,6]

In our investigation of the pharmacological activity of purified endotoxin isolated from the cholera vibrio,[7] the effect of the toxin on the permeability of living membranes to fluid has been of some interest. Frog skin from the ventral surface and small intestine of the guinea pig and rabbit have been used; other membranes such as rabbit omentum proved too fragile or otherwise unsatisfactory. Graduated columns were made from serological pipettes, usually of 5 ml capacity. The tops were cut off and flanged slightly to facilitate filling. Thistle tubes, 30 mm in diameter, were sealed on in place of the tips for the experiments with frog skin and the skin gently stretched over the opening and tied on. Short lengths of glass tubing 3 mm in diameter for guinea pig intestine and 5 mm for rabbit intestine, were sealed on in place of the tips of other pipettes. Pieces of intestine, approximately 6 cm in length, were taken from the freshly killed animal, one end slipped over the end of the graduated column and tied on and the other end tied off. The upright column was then filled with Ringer-Locke solution and the lower end and membrane immersed in the same solution in a small beaker and incubated in a water bath. The hydrostatic pressure of 8-10 inches on one side of the membrane was sufficient to produce an appreciable rate of flow across the membrane which was measured by periodic observation of the volume in the column. Measurement of the volume within the membrane and column at the beginning and end of such experiments indicated that distention of the membrane was not quantitatively important. The rate of flow was approximately linear with respect to time except for a latent period in the first 30-60 minutes (See Fig. 1). In preliminary experiments the addition of 20% sucrose to the solution in the beaker did not increase the rate of flow and was appreciably toxic to the membranes.

Skin from the ventral surface of the frog was considerably more satisfactory than the dorsal skin but the direction of flow through the skin was not important. In the case of small intestine there appeared to be no significant differences between duodenum, jejunum, and ileum. Here also the direction of flow was not material, there being no advantage and considerable disadvantage in turning the piece inside out. Peristalsis persisted for 8-10 hours at 37°C and for 18 hours or more at 24°C but no experiment with intestine was carried longer than 6-7 hours. The rate of flow did not differ appreciably over this temperature range. Experiments with frog skin were carried out at 24°C for as long as 12 hours. In view of the fact that with strips of intestine the flow was not from the lumen to the circulation but through the musculature as well, it was of interest that the rate was almost precisely the same as through frog skin. In general, however, frog skin was most satisfactory though of limited utility for our purposes, guinea pig intestine less so and rabbit intestine least satisfactory with respect to reproducibility of results. In part because of unavoidable differences in size of the pieces of intestine, there was considerable variability from one piece to another in rate of flow. When experiments were run in quadruplicate, however, results were closely reproducible.

The rate of flow of fluid through the normal membrane immersed in Ringer-Locke solution was found to be about 0.25 ml per hour. The addition of living vibrios, or crude or purified toxin to the solution either within or outside the membrane markedly accelerated this rate. Purified toxin was added as a concentrated alcoholic solution, the toxin precipitating to give an opalescent suspension in the Ringer-Locke solution; control experiments indicated that the small amount of alcohol added was not important. The acceleration of flow began to be apparent at a concentration of 0.5 MLD (Mouse) per ml and increased with increased concentration of toxin until with 4 MLD per

[6] *Cf.* Andu, A. B., and van Niekerk, J., *Centralbl. f. Bakt., I Abt. Orig.*, 1929, **112**, 519.

[7] Burrows, W., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 306.

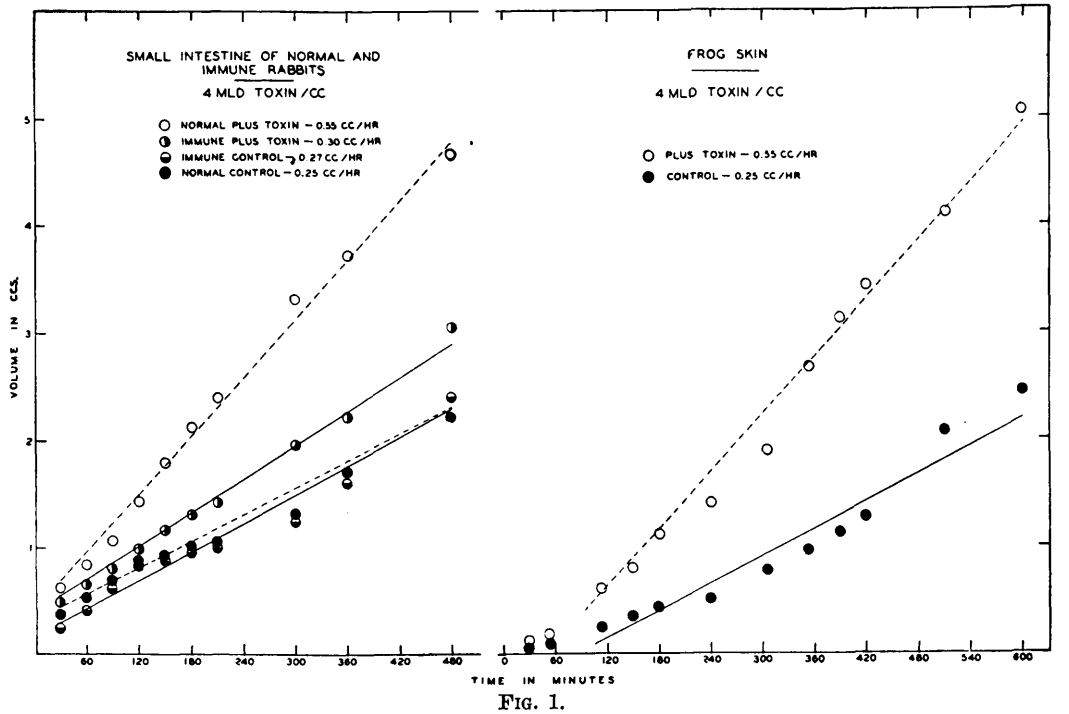

FIG. 1.

The effect of the endotoxin of the cholera vibrio on the permeability of living membranes to fluids. Left, isolated strips of intestine from normal and immune rabbits. Note that the toxin approximately doubles the rate of flow of fluid in the normal intestine, while the intestine from the immune animal does not differ significantly from the controls. Right, frog skin. Note the precise duplication of results with the normal rabbit intestine. Rates were determined graphically and lines fitted by inspection.

ml the rate was approximately double that of the controls as shown in Fig. 1. At this concentration the toxin appeared to have no deleterious effect on the membrane, and did not influence peristalsis of the strips of intestine. With higher concentrations more rapid flow could be produced—we have observed as much as 6-fold differences—but the results were inconsistent and erratic. It was immaterial whether the toxin was placed inside or outside the membrane, within or outside the lumen in the case of strips of intestine, and for the most part it was put inside because of the smaller volume and consequent economy of toxin. When crude toxin, such as ground vibrios, or vibrios precipitated with minimal amounts of trichloracetic acid, was used, it could not be detected in the outside solution at the end of the experiment by inoculation of mice with pooled and concentrated solution. Purified toxin, however, diffused through the intestine and assay by mouse inoculation indicated that by the end of the experiment the concentrations inside and outside were approximately the same. The acceleration of flow occurred only in the presence of toxin for strips of intestine soaked over night in Ringer-Locke solution containing 4 MLD of toxin per ml and thoroughly washed before use did not differ from normal controls.

When the increased permeability was produced by suspensions of living vibrios, the addition of antiserum agglutinated the bacteria and the accelerating effect was no longer apparent. The effect of endotoxin, either crude or purified, however, could not be neutralized by antiserum, even hyperimmune serum showing agglutinin titers of 1:50,000 and powerful protective properties by mouse assay, in concentrations as high as 10% in the toxin-Ringer-Locke solution. Nor were strips of intestine soaked in such immune serum solutions overnight appreciably resistant to the effect of the toxin. If, however, strips of intestine were taken from immune animals they were completely, or almost com-

pletely, resistant to the action of the toxin and in its presence showed little or no difference in permeability to fluids from normal intestine in the absence of toxin. Data from typical experiments are given in graphical form in Fig. 1.

These phenomena have been studied in detail and the investigation extended considerably further than indicated here; these studies will be reported fully elsewhere. It may be pointed out, however, that these results are consistent with and complementary to those reported in a preceding paper[8] and it is apparent that active immunity to Asiatic cholera in the experimental animal, and presumably also in man, includes antitoxic as well as antibacterial immunity. The significance of the former in enteric infection with the vibrios is clearly indicated though not as yet quantitatively evaluated. If it be assumed that the resistance of the immune intestine to the action of the endotoxin has an antigen-antibody basis, and if this is not assumed an entirely new type of immunity must be postulated, it is clear that the functional antibody cannot be circulating antibody in the isolated strip of intestine and may, therefore, be intracellular antibody, presumably in the macrophages of the tissues of the intestine.

8 Burrows, W., Mather, A. N., Wagner, S. M., and McGann, V. G., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 308.

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Influence of Citric Acid on Blood Pyruvate Levels in Man.

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Von Euler and Högberg[1] reported that the administration of citric acid produces a delayed decrease in the concentration of pyruvic acid in the blood. The principal evidence was from a small series of experiments with rats receiving oral or subcutaneous dosages of sodium citrate. Emphasis, however, was placed on a single experiment with a normal man whose blood pyruvate was lowered to 22% of the original level 12 to 15 hours after the oral ingestion of 2 g of citric acid as the sodium salt. The blood pyruvate change in this man was stated to be accompanied by indications of a profound general disturbance including sweating, shivering, and fever.

Apart from the suggestion of important theoretical relations this report is of interest because of the growing use of blood pyruvate measurements in the study of thiamine deficiency and the fact that the citric acid dosage employed is scarcely heroic. Two grams of citric acid would be supplied by one large lemon or by 200 cc of orange juice.[2]

In this Laboratory this question was studied in 5 experiments on 3 normal young men who had been maintained on a carefully standardized dietary for several months. In each experiment a blood sample was drawn before breakfast at 8:00 a.m. and 3 g of citric acid as Na citrate were administered orally in 200 cc of orange juice on the same day at 6.00 p.m. The final blood sample was taken before breakfast on the following morning at 8:00 a.m., that is, 14 hours after the citric acid administration. The blood samples were drawn from veins in the antecubital fossa. Pyruvate was estimated in duplicate by the method of Friedemann and Haugen.[3]

The results are summarized in Table I. It is clear that the citric acid had no effect of any kind on the pyruvate level at the time—14 hours—when the maximum change was

1 Von Euler, H., and Högberg, B., *Z. Physiol. Chem.*, 1940, **265**, 244.

2 Chatfield, C., and Adams, G., *U. S. Dept. Agric. Circular* No. 549, 1940.

3 Friedemann, T. E., and Haugen, G. E., *J. Biol. Chem.*, 1943, **147**, 415.