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Sonic Extraction of Labile Bacterial Constituents.

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Recent investigations have indicated that the killing of certain bacteria by available agents destroys or alters at least some of their antigenic constituents. Thus the investigations of Felix[1] have revealed a labile virulence (Vi) antigen detected only in living, virulent strains of *Eb. typhi.* Similarly, Mudd, Pettit, Lackman and Czarnetzky[2] have found that the antibody responsible for the phagocytosis of a strain of *Streptococcus hemolyticus* is elicited in rabbits by the injection of living organisms and that the living organisms will absorb the antibody from immune serum so produced. The corresponding bacterial antigen itself has resisted all ordinary methods of separation or fractionation.

Since it has been shown that sonically killed bacteria are disintegrated[3, 4, 5] and since some heat labile proteins are not denatured by the sonic treatment,[6] it appeared possible that labile but important antigens such as those referred to above might be extracted by an adaptation of the method of sonic disintegration. The purpose of this paper is to describe the apparatus and technique which we have used; to make brief mention of the antigens obtained thus far; and to point out some implications of these observations.

We have used a modified Pierce magnetostriction oscillator as the source of intense vibrations of about 8900 c.p.s. A similar oscillator has been described by Gaines[7] and was used by Chambers and Gaines[3] in their earlier work. The circuit design in our apparatus differs somewhat from that of Gaines but the essential features of the sound source are the same. The electrical circuit we have used is shown in Fig. 1.

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1 Felix, A., and Pitt, R. M., *Lancet,* 1934, **2**, 186; Felix, A., and Bhatnagar, S. S., *Brit. J. Exp. Path.,* **16**, 422.

2 Mudd, S., Pettit, H., Lackman, D., and Czarnetzky, E. J., Proc. Am. Assn. Path. and Bact., Boston Meeting, *Am. J. Path.,* 1936 (in press).

3 Chambers, L. A., and Gaines, N., *J. Cell. and Comp. Physiol.,* 1932, **1**, 451.

4 Harvey, E. N., and Loomis, A. L., *J. Bact.,* 1929, **17**, 375.

5 Williams, O. B., and Gaines, N., *J. Infect. Dis.,* 1930, **47**, 485.

6 Chambers, L. A., and Flosdorf, E. W., *J. Biol. Chem.,* 1936, **114**, 75.

7 Gaines, Newton, Physics, 1932, **3**, 209.

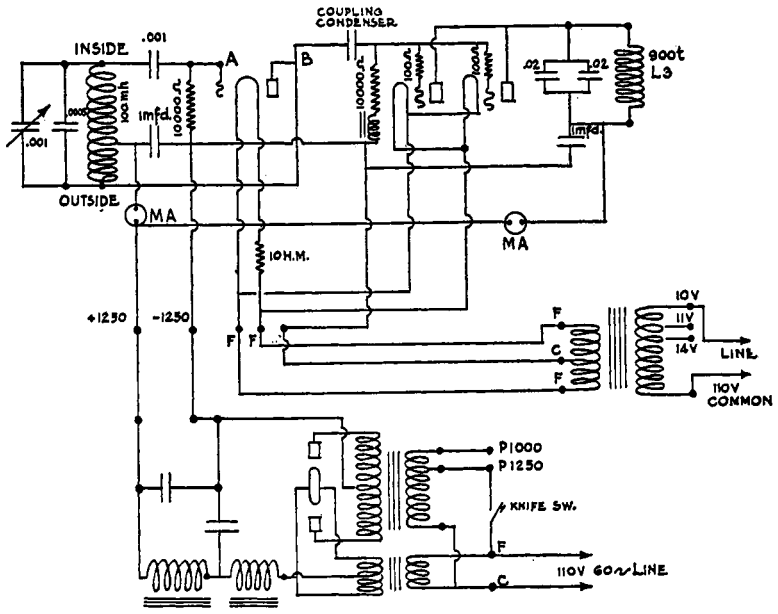

FIG. 1.
Circuit diagram of the electrical oscillator. The polarizing circuit is not included.

The arrangement of the vibrating element with relation to polarizing and driving coils is illustrated in Fig. 2. The vibrator itself is a tube of No. 20 Stubbs gauge, seamless, cold-drawn nickel tubing 24.6 cm. in length and 1.875 cm. in outside diameter. The upper end of the tube is closed by a welded nickel cap as in the original Gaines device, but we have found it unnecessary to slit the lower half of the tube. Adequate radiation of heat due to eddy currents is possible, as described below, when no such kerf is present.

The nickel tube is actuated by 1250 volt A.C. carried in the coil about the midpoint of the tube. Such a position of the driving solenoid makes possible a symmetrical distribution of the polarizing field provided by the independently excited C shaped electromagnetic circuit. The polarizing coil in our apparatus was designed to provide 5000 ampere turns, much in excess of the actual requirement. Strength of polarizing field is not critical.

Operation of the vibrating unit consists in simple tuning of the electrical oscillator to resonance with the nickel tube. In the present experiments the vibrator was of such size and shape that the system was in resonance at 8900 c.p.s. At that frequency the vibration amplitude was of the order of 0.001 cm. or just below the critical amplitude required for rupture of the nickel.

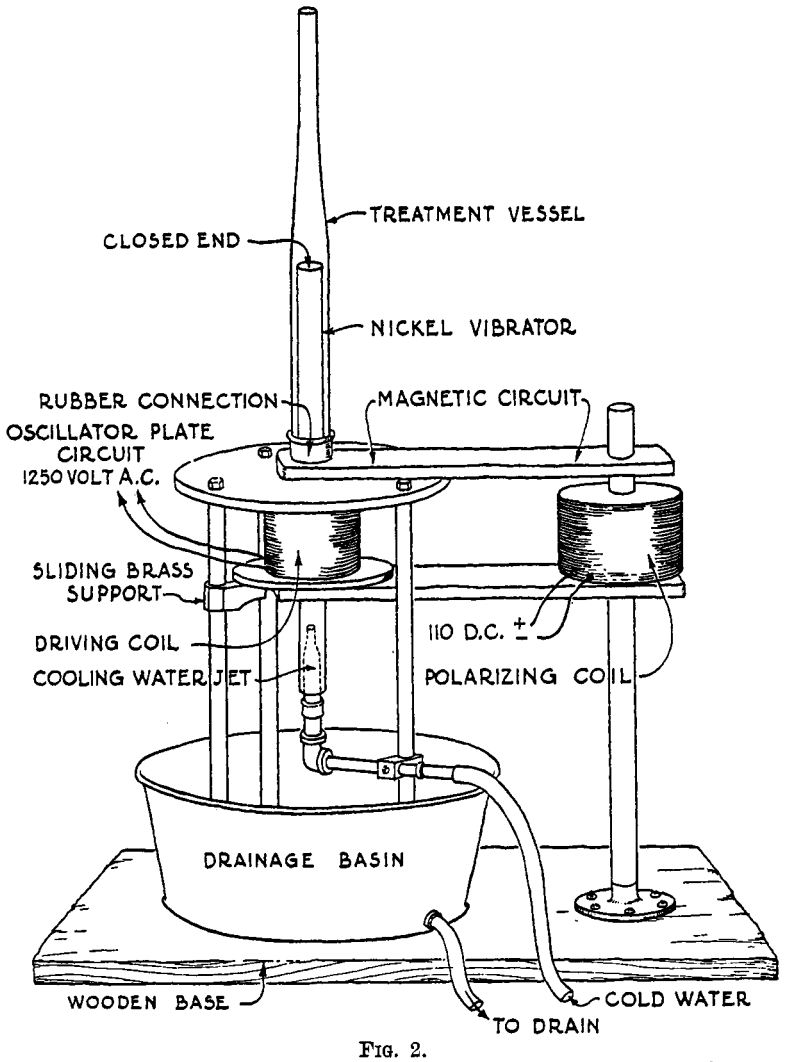

FIG. 2.

Diagram showing arrangement of driving and polarizing coils relative to the nickel vibrator. The water cooling arrangement is also represented.

Exposure of bacterial suspensions was accomplished by immersion of the capped upper end of the nickel vibrator in the suspension contained in a cylindrical glass vessel as shown in Fig. 2. The vessel was of about 2.5 cm. internal diameter and extended from the middle of the vibrator, where it was connected by a flexible rubber joint, to a point about 20 cm. above the capped end. The upper portion of the vessel was in the form of an inverted cone with an apical aperture small enough to permit convenient sterile closure,

but large enough to allow passage of a pipette. The entire assembly, nickel tube, glass vessel, and rubber joint, was easily removable from the coil support and could therefore be sterilized by autoclaving, and it could be filled and emptied under aseptic conditions.

Of primary importance in the present investigation was the problem of maintaining a low temperature within the bacterial suspension during the entire period of exposure. This was accomplished by means of a jet of cold water projected into the open lower end of the vibrating tube in a manner similar to that previously described. The temperature of the water was about 10° and an adequate flow for maintenance of a 15° level in the suspension was found to be about 2 liters per minute.

Labile constituents with antibody combining power have been identified in the supernatant liquid obtained by centrifuging sonically disintegrated *Eberthella typhi* and *Streptococcus hemolyticus*.

The *Eb. typhi* culture was a mixture of 3 virulent strains recently isolated from carriers and from a severe case. Each strain contained the "Vi" antigen. The organisms were cultured on infusion agar (pH 7.2), and the washings from 8 Roux bottles suspended in physiological saline and diluted to a total volume of 20 ml. were used for each sonic extraction. Vibration of such a heavy suspension resulted in a rapid decrease in opacity until at the end of 45 minutes the liquid was quite translucent but not completely clear. Tests subsequent to exposure showed relatively very few viable cells. Microscopically it was possible to distinguish a few intact organisms and a few recognizable bacterial fragments but the bulk of the suspended material was unorganized.

After vibration for 45 minutes the suspension was centrifuged to remove the debris and bacterial residue. With the collaboration of Mr. R. E. Greaves it has been found that the resulting clear liquid will precipitate the "Vi" antibody in sera from which all somatic and flagellar antibodies had previously been absorbed. After precipitation with the sonic material this absorbed serum will no longer cause the agglutination of organisms containing the "Vi" antigen. A full report of the identification of the extracted antigen will be published elsewhere.

Of still greater interest is the new antibody-combining constituent extracted in similar manner from *Streptococcus hemolyticus*. A virulent human strain (Griffith's Type I) in the mucoid phase and of recent animal passage was used. Organisms in 7.5 liters of 18-hour infusion broth culture were separated by centrifugalization yielding about 2 ml. of solid organisms. They were then suspended

in 18 ml. of 0.85% NaCl. The final concentration of living cells was of the order of 10^{11} per milliliter.

Such a suspension was exposed to vibration in the glass treatment vessel for one hour. It was then centrifugalized to remove the debris. By this operation about 18 ml. of clear liquid was recovered. During vibration the same clearing of the suspension noted in the case of *Eb. typhi* occurred, but to a lesser degree. Microscopic observation showed a rapid shattering and dispersion of clumps and chains, followed by a less rapid disintegration of the individual cells. The disintegrative process has been studied in detail by one of us (Chambers) and full description is in preparation.

As was true in the case of the typhoid bacillus, the streptococcus suspension was not rendered perfectly sterile by one hour of vibration. The survival curve is typically logarithmic, its slope being a function of the sound intensity—in other words, of the amplitude when the frequency is constant. Because of the lack of sterility it was necessary to pass the clear supernatant liquid rapidly through a Berkefeld filter under conditions permitting a minimum adsorption of active material. This was accomplished by dilution of the recovered 18 ml. with saline to a volume of 72 ml. before filtration.

The soluble protein contained in the filtrate will absorb from immune serum the antibody responsible for phagocytosis as shown by Mudd, Pettit, Lackman and Czarnetzky, who will publish elsewhere a full account of their work. The substance is destroyed by formalin and is inactivated at pH 10. It is also inactivated by heating for 30 minutes at 56°; similarly exposure of a suspension of living streptococci of this strain to 56° for 30 minutes suffices for sterilization. Furthermore the lability of the antigen is such that it deteriorates completely during three days of storage at 2° to 4°C. It may, however, be stored in "lyophile"[8] form after rapid drying from the frozen state and it will be very unusual if we do not find that it can be stored at least a year in this condition without deterioration.

The extraction of labile substances from living bacterial cells by sonic comminution appears to be a tool of considerable usefulness in immunology. The substances extracted thus far may prove of great practical importance if studies now under way show that the extracts will elicit antibody formation in the experimental animal. The implications are obvious. Production of antibodies against antigens that are destroyed by previously employed means of vaccine

[8] Flosdorf, E. W., and Mudd, S., *J. Immunol.*, 1935, **29**, 389.

production is of more than theoretical interest since it is these antibodies which may be of the greatest importance in combatting infection by the more virulent strains of organisms.[1, 9] On the other hand, should no results of immediate practical importance result from the extraction of the 2 labile substances, the value of the sonic method as a research tool in immunology and bacteriology has already been demonstrated. For example, known methods of mechanical disintegration, as in the ball mill, are too slow in their action to enable the extraction of constituents which deteriorate completely during three days of storage at low temperature. Only by rapid sonic disintegration combined with lyophile preservation has it been possible to obtain the new *S. hemolyticus* material in sufficiently stable form for use in experiments which require more than a day for completion.

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Myohemoglobin Content of the Hypertrophied Heart of the Anemic Rat.

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The anemia which develops in rats raised on an iron and copper-free diet is accompanied by marked cardiac hypertrophy and significant chemical changes in the myocardium. A study of the muscle hemoglobin in the hearts of these animals is presented in this paper.

Most of the methods used were described in an earlier report.[1] In the present series, the animals were quickly opened along the mid-line while under light ether anesthesia, a cannula inserted into the inferior vena cava, and the point forced up past the level of the diaphragm. The heart was thus perfused with normal saline containing 5% glucose at 37°C. until it ceased to beat. At this time, the fluid returning from the heart was practically free of blood hemoglobin as indicated by lack of color. The ventricles were then carefully severed from the body at the atrioventricular ring. They were opened immediately, blotted dry, weighed and analyzed for muscle hemoglobin by the procedure described by Whipple.[2] Blood

[9] Day, H. B., *J. Path. and Bact.*, 1933, **37**, 169.

[1] Cowan, D. W., PROC. SOC. EXP. BIOL. AND MED., 1934, **31**, 417.

[2] Whipple, G. H., *Am. J. Physiol.*, 1926, **76**, 693.