pancreatic enzymes; it is inactivated by incubation with serum, probably on an enzyme basis.

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CHLORELLIN, AN ANTIBACTERIAL SUBSTANCE FROM CHLORELLA

CULTURES of *Chlorella,* in inorganic nutrient solutions, produce and accumulate a substance that tends to inhibit further multiplication of the cells.[1] Recently extracts containing this growth-inhibiting substance have been prepared in larger quantities than heretofore and have been tested for antibiotic activity against other organisms. These extracts have been found to possess antibacterial properties against both Gram-positive and Gram-negative organisms: *Staphylococcus aureus, Streptococcus pyogenes, Bacillus subtilis, Bacterium coli* and *Pseudomonas pyocyanea* (*Ps. aeruginosa*).

Both *Chlorella vulgaris* and *Chlorella pyrenoidosa,* commonly considered to be different species, were used in these experiments. The cells were cultured in batteries of 5-gallon bottles containing solutions of the conventional mineral nutrients for pure algal cultures. Various proportions and concentrations of these salts were tested. The more dilute solutions, and those containing ammonium nitrogen, were all sterilized before inoculation. With more concentrated solutions,[1] sterilization was not essential when the solutions were continuously illuminated. A mixture of 5 per cent. carbon dioxide in air was bubbled continuously through the cultures. Some cultures were grown under continuous illumination from white fluorescent lamps for about two weeks. Others were grown in a greenhouse under natural illumination for approximately one month, finally being subjected to continuous illumination from fluorescent lamps for two to three days. Best yields of antibacterial substance were obtained from mature cultures which were harvested immediately after the above mentioned period of illumination.

Various methods have been used to obtain extracts containing the active principle. Crude extracts, suitable for determining the biological activity of the inhibitory substance, were prepared by extraction of the cell-freed culture solutions. For these extractions chloroform, 1,2-dichloroethane or benzene have proven superior to other solvents tried. The separated organic solvent, used for extraction, was removed *in vacuo* and the crude extract was obtained as a brown mass, in yields of 1 to 8 mg per liter of cell-free solution. The consistency of the crude extracts varied from a viscous, tacky liquid to a brittle solid. In some instances, extraction of the cell-freed culture solution was preceded by concentration to one half volume by distillation *in vacuo* at bath temperatures of 50–55° C. Thus far adsorption and elution techniques have been inferior to the extraction procedure. Active material has also been obtained from the cell mass. Apparently moderate heat does not greatly affect the yields of the active material.

Biological tests were carried out on clear aqueous extracts of the crude material. To this end the crude material was thoroughly shaken with a small amount of water. Such a procedure has commonly yielded solutions containing 0.03 to 0.1 mg solids per cc. This solution was adjusted to pH 7.0. When tested against *Staphylococcus aureus,* strain 209, in standard cup assay (18 hours at 37° C., diameter of cup 8 mm) 0.2 cc portions of these solutions commonly produced zones of inhibition 18 to 35 mm in diameter. A zone of 45 mm was obtained from extracts of one lot of crude material. Attention should be directed to the fact that only a very small portion of the active principle is extracted in a single treatment by this procedure, for repeated extractions of a given lot of crude material continue to yield solutions showing a relatively high order of antibacterial activity. The same order of activity was observed when strains of *Str. pyogenes* (on blood agar) or of *B. coli* were substituted as the test organism. The strain of *Ps. pyocyanea* that was used is only slightly less sensitive to the action of this antibacterial agent, while the strain of *Bac. subtilis* is more sensitive than the staphylococcus.

Other tests indicate that the active principle may be bactericidal. One tenth of a cubic centimeter portions of 24-hour broth cultures of *Staph. aureus* were added to 5 cc portions of solutions having the same concentration of active principle as were used in the cup tests. Less than 10 minutes contact with the active principle prevented subsequent multiplication of the organisms when transferred to nutrient broth (incubated at 37° C. for 48 hours). Contact of about 20 minutes was required in the case of *B. coli* to achieve the same result. Similar tests in which the bacterial cells were exposed to the solution of the active principle in fresh rabbit serum showed that the bactericidal activity of the active principle is but slightly inhibited in the presence of serum proteins.

The results of numerous experiments carried out over a period of a year and a half show that an antibacterial substance accumulates in uncontaminated cultures of *Chlorella* and that the activity of this substance can be tested by standard bacteriological methods. For convenience of reference it is proposed to designate this substance by the name *chlorellin.* It is recognized that the products thus far obtained repre-

[1] R. Pratt, *Am. Jour. Bot.,* 27: 52 and 431, 1940; 29: 142, 1942.

sent crude extracts. Considerable work will be required to determine fully the range of biological activity and the chemical nature of the active principle. In view of the low and variable yields thus far obtained it is apparent that further work is necessary to determine the optimum conditions for the accumulation and extraction of chlorellin. The cell mass may prove to be a valuable source of the active principle. These investigations are progressing.

Chlorellin is unique in the constantly growing list of antibiotics which have been reported in the literature, because, since it is derived from an autotrophic organism, its production does not entail the use of expensive and troublesome organic culture media, only inorganic salts, carbon dioxide and light being required.

These investigations have been carried out as a cooperative project by staff members of the College of Pharmacy of the University of California and of the Division of Plant Biology of the Carnegie Institution of Washington.

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SCIENTIFIC APPARATUS AND LABORATORY METHODS

NEW INDICATORS TO REPLACE LITMUS IN MILK

THE use of litmus milk has gained wide popularity in bacteriological procedures because of the large number of biochemical reactions that can be determined with one inoculation. It has been our experience to find it very difficult to duplicate the density of the color. This may be due to the fact that litmus is not a single substance but a mixture which varies with the lichen and preparation used.

A search for a single compound which would serve both as a pH indicator and an E_h (oxidation-reduction) indicator in the proper ranges proved disappointing. A combination of indicators was decided upon, and those embodying the most desirable properties both alone and in combination were Chlor Phenol Red (or Brom Phenol Red) for the pH indicator and Methylene Blue for the E_h indicator.

Chlor Phenol Red is a member of the sulfonphthalein dyes which are widely used in bacteriological media. The pH range is from 5.2 to 6.8. Its yellow color on the acid side and red color on the basic side does not mask the reactions of the Methylene Blue. A stock solution is prepared by dissolving 0.1 gram in 10 cc of absolute alcohol.

The Methylene Blue stock solution is prepared by dissolving 0.625 grams (total dye concentration 84 per cent.) in 250 ml of distilled water. The stock solutions are kept in well-stoppered light-tight bottles.

The formula for the preparation of the milk is as follows:

Dry milk powder	90 grams
Chlor Phenol Red (stock solution)	1 ml
Methylene Blue (stock solution)	2 ml
Water	make up to 1 liter

The milk is then sterilized by the fractional method or autoclaved at 15 pounds for 15 minutes. The color of the milk changes to a light pink when strongly heated, but the original color returns as the milk cools.

Autoclaved milk requires a somewhat longer time for acid coagulation, but this is foretold by the very rapid reduction of Methylene Blue in this reaction.

The color changes in the milk as compared with Ridgeway's Color Standards (1912) are summarized as follows:

Uninoculated	Pale Glaucous Green
Slightly acid	Yellowish Glaucous
Acid with reduction	Ivory Yellow
Alkaline	Pale Russian Blue
Alkaline with reduction	Pale Pinkish Cinnamon
Alkaline peptonization	Clear Transparent Red

Inoculation of organisms of known biochemical character readily overcomes confusion in transposing the same reactions found on litmus milk.

This medium has been successfully used in a number of beginning and advanced classes in bacteriology. Students using milk for the first time have no difficulty in determining the reactions which have taken place.

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