

## Effect of Chlorophyll Derivatives and Related Compounds on the Growth of *M. tuberculosis*.\*

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Attenuation in the virulence of the bovine tubercle bacillus by cultivation on ox-bile media (Calmette and Guérin,<sup>1</sup> Bouquet and Nègre<sup>2</sup>) suggests the evaluation of the individual constituents of bile as to their possible effect on *M. tuberculosis*. However, of the many bile constituents, only certain bile acids and their salts have been studied in detail regarding their action on the tubercle bacillus (Proskauer and Beck,<sup>3</sup> Deyke and Much<sup>4</sup>). Present in bile are certain decomposition products of chlorophyll, particularly phyloerythrin (Loebisch and Fischler<sup>5</sup>) and pyrroporphyrin (Rothemund<sup>6</sup>). It was therefore considered desirable to determine whether certain water soluble chlorophyll derivatives and related porphyrins affected the growth of these organisms.

The chlorophyll compounds, selected because of their solubility in water, were chlorin-e, the copper complex of chlorin-e, and chlorophyllin-a. Besides these compounds, closely related to chlorophyll, certain porphyrins were studied—pyrroporphyrin, representing the chlorophyll porphyrins and deuteroporphyrin, representing the blood porphyrins.

Chlorophyllin-a was prepared following Willstaetter's<sup>7</sup> method, by alkaline cleavage of chlorophyll-a. It is a tri-carboxylic acid in which two of the carboxyl groups occur as in the original chlorophyll molecule, while the third one is formed by the splitting of the isocyclic ring. To make chlorophyllin water soluble, the tri-sodium salt was prepared.

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<sup>1</sup> Calmette, A., and Guérin, C., *Ann. d. l'Inst. Past.*, 1908, **22**, 689.

<sup>2</sup> Bouquet, A., and Nègre, L., *Ann. d. l'Inst. Past.*, 1921, **35**, 142.

<sup>3</sup> Proskauer, B., and Beck, M., *Z. f. Hyg.*, 1894, **18**, 128.

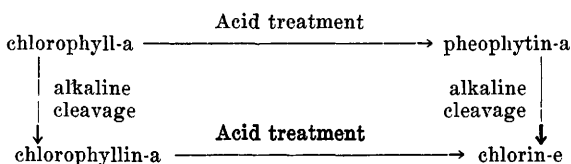
<sup>4</sup> Deyke, G., and Much, H., *Munch. Med. Woch.*, 1909, **56**, 1985.

<sup>5</sup> Loebisch, W. F., and Fischler, M., *Monatsch. f. Chemie*, 1903, **24**, 335.

<sup>6</sup> Rothemund, P., *J. Am. Chem. Soc.*, 1935, **57**, 2180.

<sup>7</sup> Willstaetter, R. M., *Untersuchungen Ueber Chlorophyll*, J. Springer, Berlin, 1913.

Chlorin-e is prepared by splitting off the magnesium atom from chlorophyllin by acid treatment. However, it is more convenient to prepare it from pheophytin-a, a compound which corresponds to chlorophyll, but is magnesium-free. Alkaline cleavage of pheophytin-a, following Willstaetter's process, yields chlorin-e in the same way as chlorophyll, with alkaline treatment, yields chlorophyllin. Here again the trisodium salt of chlorin-e was prepared to make the compound water-soluble.



The metal complex of chlorin-e, copper chlorin-e, was made and rendered water soluble in the same way as the metal-free chlorin-e. The copper atom is bound in the center of the molecule as magnesium is found in chlorophyllin.

Pyrroporphyrin is a chlorophyll porphyrin obtainable by alkaline cleavage of pheophytin at higher temperatures. It is a mono-carboxylic acid retaining the original propionic acid group of the chlorophyll molecule; it has one carbon atom in the nucleus which is saturated with hydrogen and not substituted. The water soluble derivative, pyrroporphyrin sulfonic acid was prepared by sulfonation of the carbon atom with pyridinium-N-sulfonic acid according to the method of Treibs.<sup>8</sup>

Deuteroporphyrin is a blood porphyrin with 2 propionic acid groups and with 2 unsubstituted nucleus carbon atoms. It is obtained by reduction of hemin with resorcinol following Schumm's<sup>9</sup> method. In order to make the compound water soluble, the di-sulfonic acid derivative was prepared by sulfonation with pyridinium-N-sulfonic acid (Treibs). The 2 sulfonic acid groups are bound to the unsubstituted nucleus carbon atoms. This deuteroporphyrin di-sulfonic acid, having 4 acid groups of which 2 are the original propionic acid groups of the deuteroporphyrin and 2 are formed by sulfonation, is water soluble. The tetra sodium salt was prepared.

The copper complex of this compound has the copper atom bound in the same way as the metal is bound in copper chlorin-e.

These compounds are readily identified in solution by their characteristic absorption spectra.

<sup>8</sup> Treibs, A., *Annalen d. Chemie*, 1933, **506**, 196.

<sup>9</sup> Schumm, O., *Z. f. Phys. Chemie*, 1928, **178**, 11.

Compound	Absorption spectrum		Sequence of Intensities
	Maxima $\mu\mu$		
chlorophyllin-a tri-sodium salt	639, 595, 530	I, II, III	
chlorin-e tri-sodium salt	653, 598, 543, 503	I, IV, II, III	
chlorin-e, copper tri-sodium salt	629, 585, 503	I, III, II	
pyrroporphyrin sulfonic acid sodium salt	631, 575, 554, 514	III, IV, II, I	
deuteroporphyrin di-sulfonic acid tetra sodium salt	627, 613, 572, 549, 512	III, V, IV, II, I	
deuteroporphyrin copper disulfonic acid tetra sodium salt	573, 537	I, II	

For further information concerning these compounds, the reader is referred to the original works of Hans Fischer or to the comprehensive reviews by Steele<sup>10</sup> and by Fischer.<sup>11</sup>

*Method.* Twenty-five mg amounts of the various compounds were dissolved in 100 cc portions of 4% glycerine broth or Sauton's medium in 250 cc Erlenmeyer flasks and the solutions sterilized in the Arnold for 45 minutes on 2 successive days. The pyrroporphyrin sulfonic acid was practically insoluble in broth and Sauton's medium after sterilization. The final pH of the media was determined by glass electrode. All pH values were between 7.15 to 7.60. The flasks were inoculated with young actively growing pellicles of avian or human-37 tubercle bacilli and incubated at 37°C in the dark for varying periods (21-42 days). Each compound was tested in a total of 12 flasks on three different occasions.

*Results.* Examination of the data shows that the chlorin-e and chlorophyllin-a definitely retarded the growth of both the avian and human strains. The inocula on the media containing these 2 compounds showed very slight filmy growths while the control cultures and those containing copper chlorin-e, deuteroporphyrin, copper deuteroporphyrin, pyrroporphyrin showed luxuriant growths covering the whole surfaces of the flask and encroaching on the side walls. In order to determine whether greater concentrations of the various compounds would have any effect, inoculations were made into media of 100 cc volume containing 50 mg of compound. The results were the same as those with the smaller concentration (See Fig. 1).

To test the viability of the bacilli which had been incubated in the chlorin-e and chlorophyllin flasks, transplants were made into control media not containing chlorophyll. Normal luxuriant growth of the avian strain occurred within 9 days and the human-37 showed good growth in 16 days, indicating that the compounds were bacteriostatic but not bactericidal in the concentrations used. Both organisms on

<sup>10</sup> Steele, C. C., *Chem. Rev.*, 1937, **20**, 1.

<sup>11</sup> Fischer, H., *Chem. Rev.*, 1937, **20**, 41.

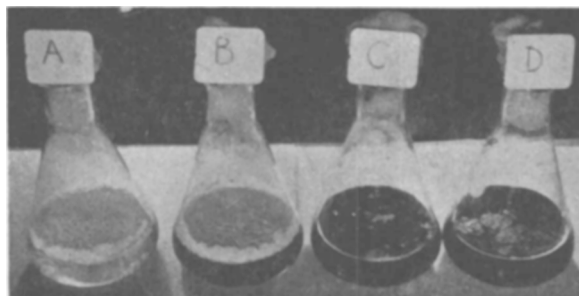


FIG 1.

Photograph shows amount of growth of *M. tuberculosis* strain H-37 on 4% glycerine broth containing:

- A—No compound (control).
- B—.025 % copper chlorin-e.
- C—.025 % sodium chlorin-e.
- D—.025 % sodium chlorophyllin-a.

all media retained their original morphological and acid-fast properties.

The pellicles assumed the color of the compounds on which they were grown (green in the case of chlorin-e and chlorophyllin and pink in the deuteroporphyrin). In order to determine in which way the pigment associates with the bacterial cell, the green pellicles were subjected to the extraction technics of Anderson<sup>12</sup> and of Heidelberg<sup>13, 14</sup> and collaborators. To ascertain whether the chlorophyll derivative was contained in the lipid fraction of the bacillus, the green pellicles of H-37 grown on copper chlorin-e were, after washing with distilled water and drying, extracted with acetone, following Anderson's procedure, to remove the lipoids. Practically none of the color was removed with the lipid extract, the organisms remaining green.

To facilitate the extraction of the proteins, the defatted organisms were ground in a ball mill until no bacilli remained intact, as determined by microscopic examination. The proteins were extracted by alkaline solutions, both N/10 and N/20 KOH removed the color completely. Addition of acetic acid to these alkaline extracts precipitates a green material which for purification may be redissolved in N/10 or N/20 KOH and reprecipitated by addition of acetic acid. The green compound gives a positive protein test with Millon's reagent. It has an absorption spectrum, the bands of which, compared to those of aqueous solution of copper-chlorin-e-sodium are shifted

<sup>12</sup> Anderson, R. J., *Physiol. Rev.*, 1932, **12**, 172.

<sup>13</sup> Heidelberg, M., and Kendall, F. E., *J. Exp. Med.*, 1931, **54**, 513.

<sup>14</sup> Heidelberg, M., and Menzel, A. E. O., *Proc. Soc. Exp. Biol. and Med.*, 1932, **20**, 631; *J. Biochem.*, 1934, **104**, 655.

to the red end of the spectrum for approximately  $10\mu\mu$ . This phenomenon of shifting of the absorption bands for 10 to  $15\mu\mu$  towards the longer wave lengths is a typical and well recognized property of chlorophyll protein complexes as has been shown by Smith, French and earlier investigators. As a result of the observations of these authors, the union of chlorophyll and protein is already well recognized as a naturally existing combination, chlorophyll of higher plants (Smith<sup>15</sup>), bacterio-chlorophyll of purple bacteria (French<sup>16</sup>).

*Conclusions.* Chlorin-e and chlorophyllin in concentrations of .025 and .050% in glycerine broth and Sauton's media inhibited the growth of H-37 and avian tubercle bacilli. Copper chlorin-e, deuteroporphyrin, copper deuteroporphyrin, and pyrroporphyrin sulfonic acid-sodium salt showed no growth retarding effects in concentrations of .025 and .050%. Evidence is presented to suggest that the H-37 tuberculo-protein of the living bacillus is capable of binding copper chlorin-e. The absorption spectrum of the copper chlorin-e-bacillary protein complex when compared with that of copper chlorin-e-sodium in aqueous solution lends support to the assumption that chemical union between protein and pigment exists.

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### Mosquito Transmission Experiment with Poliomyelitis Virus.

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Although a few attempts have been made to transmit the virus of acute anterior poliomyelitis by mosquitoes, the field does not appear to have been entirely exhausted of possibilities and the following is submitted as a slight contribution to this line of investigation. Simmons, Kelsner and Cornell<sup>1</sup> have summarized the earlier efforts and this summary will not be repeated. Theoretical arguments for or against the possibility will decide nothing, but repeated efforts may add weight to either side of the question.

<sup>15</sup> Smith, E. L., *Science*, 1938, **88**, 170.

<sup>16</sup> French, C. S., *Science*, 1938, **88**, 2272; *J. Gen. Physiol.*, 1937, **20**, 711; *ibid.*, 1937, **21**, 71.

<sup>1</sup> Simmons, J. S., Kelsner, R., and Cornell, V. H., *Proc. Soc. Exp. Biol. and Med.*, 1934, **31**, 496.