ON THE INHIBITORY INFLUENCE OF EOSIN UPON SPORULATION.[1]

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Sporulation requires optimum temperature and suitable nutrient media. It is greatly influenced by various physical and chemical agents. Thus, in the case of Bacillus anthracis, the most studied in this respect of all spore-bearing organisms, no sporulation takes place at temperatures above 42° C.[2] or below 14° C.[3] Weil[4] places the lowest temperature at which sporulation takes place at 7° C. The presence of oxygen seems to be essential to the formation of anthrax spores.[5] Persistence of conditions unfavorable to sporulation through many successive generations of the organism gives rise to asporogenous strains in which virulence is often found greatly reduced or even totally absent.

Phisalix[6] succeeded in obtaining an asporogenous stain of B. anthracis by cultivating it at 42° C. for twelve successive generations. Roux[7] induced a similar biological alteration by means of a medium containing certain chemicals. Potassium bichromate in the ratio of 1 to 2000, or phenol in from 2 to 6 to 10,000 added to ordinary bouillon stops sporulation completely. Behring[8] found that various acids, alkalies, salts and certain antiseptics when used in suitable concentrations prevent sporulation. The antisporulative property of certain dyes was also described by Behring,[9] who states that safranin in 1 to 30,000, and methylene violet, cyanid, and malachite green in 1 to 200,000 to 1 to 600,000 exert a powerful restraining influence upon the growth and sporulation of B. anthracis. After

[1] Received for publication October 1, 1907.

[2] Phisalix, *Bull. méd.*, 1892, vi, 533.

[3] Kitasato, *Zeit. f. Hygiene,* 1890, viii, 198.

[4] Weil, *Zeit. f. Hygiene,* 1901, xxxvi, 451.

[5] Schreiber, *Cent. f. Bakt.,* 1896, xx, 353. Weil, *Arch. f. Hygiene,* 1901, xxxix, 205. Klett, *Zeit. f. Hygiene,* 1900, xxx, 420. Jacobitz, *Cent. f. Bakt.,* 1901, xxx, 232. Slupski, *Cent. f. Bakt.,* 1901, xxx, 396.

[6] Phisalix, *Bull. méd.*, 1892, vi, 533.

[7] Roux, *Annales de l'Inst. Pasteur,* 1890, iv, 25.

[8] Behring, *Zeit. f. Hygiene,* 1889, vi, 117.

[9] *Idem,* 1889, vii, 171.

two months' successive cultivation in the colored agar media, no permanent loss of the sporulating property resulted.

Schreiber[10] states that potassium phosphate of a concentration above 3 per cent. prevents sporulation of B. anthracis, B. subtilis and B. tumescens. Behring,[11] Bormans,[12] and Lecleff[13] found that B. anthracis does not form spores in blood serum, while Brieger, Kitasato and Wasserman[14] recorded a few instances in which B. tetani failed to sporulate in an aqueous extract of thymus gland.

While abundant work has been done with various spore-bearing aerobes, especially with B. anthracis, a similar study with anaerobic organisms has been so far neglected. In a recent study on the antitetanic property of certain dyes, Flexner and Noguchi[15] called attention to the fact that eosin in an adequate strength prevents the sporulation of B. tetani, the experimental details of which are given in a later paper by Noguchi.[16]

In the present communication I wish to present some of the results of experiments on the restraining influence of eosin upon the sporulation of various microbes. The varieties of bacteria subjected to experiment belonged to the aerobic and to the anaerobic organisms. Of the first, B. anthracis, B. megatherium, B. cereus, B. mesentericus, B. subtilis, B. ruminatus, and B. anthracoides, and of the second, B. tetani, B. anthracis symptomaticus, B. botulismus, B. œdema maligni, B. enteritidis sporogenes and B. putrificus were studied.

Eosin "Gelb" having been mixed with agar or bouillon in varying strengths, the inoculations of bacteria were made as usual. Stab and slant solid cultures were made. For the anaerobic bacteria, tissue-bouillon and a deep layer of glucose agar were employed. The agar tubes were incubated in an atmosphere of hydrogen. The results are tabulated.

Table I. indicates that the inhibitory action of eosin is most intense upon B. cereus and B. mesentericus, and least upon B. anthracoides, while B. subtilis, ruminatus, anthracis and megatherium occupy intermediary positions. All growth became uncertain when the concentration of eosin reached one per cent.; below this

[10] Schreiber, *Cent. f. Bakt.*, 1896, xx, 353.
[11] Behring, *Zeit. f. Hygiene*, 1889, vi, 117.
[12] Bormans, cited in *Baumgarten's Jahresberichte*, 1895, xi, 138.
[13] Lecleff, *La Cellule*, 1894, x, 349.
[14] Brieger, Kitasato and Wassermann, *Zeit. f. Hygiene*, 1892, xii, 137.
[15] Flexner and Noguchi, *Jour. of Exper. Med.*, 1906, viii, 1.
[16] Noguchi, *Jour. of Exper. Med.*, 1907, ix, 281, 291.

TABLE I.—*Examination after 10 Days.*

Slant Agar with Eosin "Gelb."	B. cereus.	B. mesentericus.	B. subtilis.	B. ruminatus.	B. anthracoides.	B. anthracis.	B. megatherium.
Control (no eosin).	+ all	+ all	+ all	+ all	+ all	+ all	+ all
0.001 per cent.	+ few	+ few	+ many	+	+	+	+
0.01 "	—	—	+ few	+	+	+	+
0.05 "	—	—	—	+?	+	+?	+?
0.1 "	—	—	—	—	+	—	—
0.5 "	—	—	—	—	—	—	—
1 "	—	—	—	—	—	—	—
2 "	—	—	—	—	—	—	—

+ = spore formation.
— = no spore formation.
+? = sporulation doubtful.

strength, multiplication of the bacteria still takes place. These organisms, when cultivated in bouillon containing varying amounts of eosin "Gelb," appear to be even more sensitive to the action of the dye than when grown on a slant agar surface. But when the cultures are allowed to stand for many weeks, sporulation takes place in a medium containing as high concentration of the dye as 0.1 per cent. Concentrations above 0.3 per cent. prevent sporulation, to which effect is added restraint of growth and formation of chains of bacteria through imperfect multiplication. At times, marked degrees of involution occur. In no instances was growth discovered in bouillon containing two per cent. of the eosin.

In deep stab cultures the results were practically identical with those given in the table. Transplantation of the asporogenous bacilli into eosin-free media was associated with immediate return of the spore-bearing power. Many weeks' contact of the sporeless vegetative bacilli with the eosin exerted no enduring effect on the sporogenous property.

The influence of eosin "Gelb" on the sporulation of the anaerobic species has been shown in Table III. Recapitulated, they show that

TABLE II.

Bouillon Culture with Eosin "Gelb."	B. cereus.	B. mesentericus.	B subtilis.	B. ruminatus.	B. anthracoides.	B. anthracis.	B. megatherium.
Control (no eosin)	3 d. + 38 d. +	3 d. + 38 d. +	3 d. + 38 d. +	3 d. + 38 d. +	3 d. + 38 d. +	3 d. + 25 d. +	3 d. + 25 d. +
0.001 per cent.	3 d. + 38 d. +	3 d. + 38 d. +	3 d. + 38 d. +	3 d. + 38 d. +	3 d. + 38 d. +	3 d. + 25 d. +	3 d. + 25 d. +
0.003 per cent.	3 d. + 38 d. +	3 d. + 38 d. +	3 d. + 38 d. +	3 d. + 38 d. +	3 d. + 38 d. +	3 d. — 25 d. +	3 d. — 25 d. +
0.01 per cent.	3 d. — 38 d. +	3 d. + 38 d. +	3 d. + 38 d. +	3 d. + 38 d. +	3 d. + 38 d. +	3 d. — 25 d. + few	3 d. — 35 d. +
0.03 per cent.	3 d. — 38 d. +	3 d. + 38 d +	3 d. — 38 d. +	3 d. + 38 d. +	3 d. + 38 d. +	3 d. — 25 d. + few	3 d. — 25 d. +
0.1 per cent.	3 d. — 38 d. +	3 d. — 38 d. +	3 d. — } No surf. 38 d. + } membr.	3 d. — 38 d. +	3 d. — 38 d. +	3 d. — 25 d. + ?	3 d. — } No surf. 25 d. — } membr.
0.3 per cent.	3 d. — 38 d. —	3 d. — 38 d. —	3 d. — } No surf. 38 d. + } membr.	3 d. — 38 d. —	3 d. — 38 d. —	3 d. — 25 d. — ?	3 d. — } No surf. 25 d. — } membr.
1 per cent.	No growth	3 d. — 38 d. —	3 d. — 38 d. —	3 d. — 38 d. —	3 d. — 38 d. —	3 d. — 25 d. —	No growth
	No growth	No growth.	No growth	No growth	No growth	No growth	No growth

d = day or days. + = spore formation. — = no spore formation.

TABLE III.—*Observations made after 29 Days on Certain Anaerobes Cultivated Aerobically in the Presence of Tissue.*

Bouillon Containing a Small Piece of Rabbit's Fresh Liver.	B. tetani.	B. anthracis symptomaticus.	B. botulismus.	B. oedema maligni.	B. enteritidis sporogenes.	B. putrificus.
Percentage of eosin "Gelb" in the bouillon						
Control (no eosin)	+	+	+	+	+	+
0.001 per cent.	+	+	+	+	+	+
0.003 per cent.	+	+	+	+	+	+
0.01 per cent.	—	+?	+	—?	—	—
0.03 per cent.	—	—	—	—	—	—
0.1 per cent.	—	—	—	—	—	—
0.3 per cent.	—	—	—	—	—	—
1 per cent.	No growth	No growth	No growth	No growth	No growth	No growth

+ = spore formation.
— = no spore formation.
? = spore formation doubtful.

the eosin in the strength of one per cent. completely inhibits all multiplication of the bacteria. The results are only very slightly different in the case of the cultivation of the anaerobic species freely in the air, in the presence of tissue (Table III.) and in an atmosphere in hydrogen in deep glucose agar. The phenomena of restraint are less pronounced in the latter media.

SUMMARY.

Sporulation of B. anthracis, B. subtilis, B. cereus, B. ruminatus, B. mesentericus, B. anthracoides and B. megatherium does not take place in an agar medium containing eosin "Gelb" in a concentration exceeding 0.5 per cent. In a concentration of 0.1 per cent. most of these bacteria fail to produce spores. The greatest sensitiveness is shown by B. cereus and B. mesentericus. In a bouillon medium sporulation is likewise inhibited by eosin, but after a longer time—seven weeks or more—sporulation still occurs where the concentration of the dye equals one-tenth per cent.

Sporulation of B. tetani, B. anthracis symptomaticus, B. botu-

lismus, B. œdema maligni, B. enteritidis sporogenes, and B. putrificus does not take place in a medium containing eosin "Gelb" in concentrations exceeding 0.03 per cent. With these organisms, no difference was noted in the final effect, depending on the medium employed. No permanent loss of power to produce spores ensues with the bacteria tested even after long sojourn in the eosinized media.

It may be stated that on the whole the inhibitory action of eosin was more pronounced upon the anaerobic than upon the aerobic species of bacteria employed in these experiments.