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THE NATURE OF THE ANTITETANIC ACTION OF EOSIN.

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In a previous publication[1] Dr. Flexner and I gave the results which we obtained from a study of experimental tetanus in rats and guinea pigs subjected to the influence of certain photodynamic anilines. To recall briefly the main facts brought out there, it may be stated that a solution of eosin, of a certain concentration, destroys in vitro the haemolytic and tetanospastic principles of tetanus toxin, and when applied to the site of inoculation of tetanus spores on threads, or tetanus-toxin, either entirely prevents the appearance of the tetanic symptoms, or delays the appearance and diminishes the effect, and, in rats, sometimes prevents the fatal issue. In a great number of instances in which the eosin is applied directly to the spore-infected area in rats, the lives of the animals can be saved. The next step in this investigation was the determination of the mode of action of the eosin through which the effects already described are produced.

The present paper deals with the manner of action of eosin upon *Bacillus tetani* without and within the body of animals. The subject falls naturally, therefore, into the two main divisions of the action of eosin upon the tetanus bacillus *in vitro* and *in vivo*.

THE ACTION OF EOSIN UPON THE TETANUS BACILLUS IN VITRO.

Relating to this topic, the following points were considered: (1) the influence of eosin upon the germination, growth, sporulation, morphology, and toxin production of *B. tetani*; (2) the bactericidal and sporicidal effects of eosin upon *B. tetani*; (3) the viability of *B. tetani* in eosinized media; (4) the toxin-producing power of *B. tetani* exposed to the action of eosin.

[1] Flexner and Noguchi, *Jour. of Exper. Med.*, 1906, viii, 1.

Cultivation of Bacillus tetani in Eosinized Culture Media. (*a*) *Eosinized Glucose Bouillon.*—Two kinds of eosin—"rein" and "gelb"—were employed in strength of 0.001 per cent., 0.01 per cent., 0.1 per cent., and 1 per cent. A vigorous culture of the tetanus bacillus was inoculated and incubated at 37° C. in an atmosphere of hydrogen for 10 days.

The control cultures were abundant; they contained typical bacilli forming few threads and showing few spores. The growth in 0.001 per cent. eosin was equal to the control, but microscopically the thread forms predominated and no spores whatever could be found. The growth in 0.01 per cent. eosin was equal to the control, but upon microscopic examination only asporogenous thread forms were seen. The growth in 0.1 per cent. eosin was slight and showed single, asporogenous bacilli. No growth whatever took place in the 1 per cent. eosin medium.

The first effect which the eosin produces, apparently, is to suppress sporulation of the bacilli, and the next is to increase thread formation. The limit of concentration of eosin which permits restricted germination is between 0.1 and 1 per cent., and is probably not far from the former concentration. A later determination placed this limit at 0.2 per cent. The cultures prepared as described were filtered through porcelain and tested for toxicity upon rats. The number of m. l. d. per cubic centimeter of filtrate was as follows:

Control	=	1000	m.l.d.
0.001 per cent. eosin	=	700	m.l.d.
0.01 per cent. eosin	=	100	m.l.d.
0.1 per cent. eosin	=	1	m.l.d.
1.0 per cent. eosin	=	0	m.l.d.

No remarkable difference in the properties of the two kinds of dye was noticed. The reduction in toxicity is greater than the reduction in growth caused by the eosin. The diminished toxicity has, doubtless, a two-fold origin: it arises from the diminished multiplication of the bacillus, and from the injury exerted by the eosin upon the toxin originally produced. The degree of this destruction can be deduced from the relative strengths of the filtrates from the control and the 0.001 per cent. eosin cultures, and it amounts to about 30 per cent. As the eosin strength grows, the effects upon the multiplication and toxicity increase, but not in equal ratio. A corresponding comparison of the hæmolysin (tetan-

olysin) present in the several filtrates was also made. Each cubic centimeter contained the following units:

	Control filtrate		contained		*C.H.D.	20	*M.H.D.	333
0.001	per cent.	"	"rein"	"	"	12	"	242
0.001	"	"	"gelb"	"	"	10	"	200
0.01	"	"	"rein"	"	"	4	"	66
0.01	"	"	"gelb"	"	"	3	"	50
0.1	"	"	"rein"	"	"	0	"	0
0.1	"	"	"gelb"	"	"	0	"	0

* C.H.D. = complete hæmolytic dose; M.H.D. = minimal (trace) hæmolytic dose.

Eosin destroys or suppresses the tetanolysin in the cultures as it does the tetanospasmin, a fact which could be predicted on the basis of the observations of Flexner and Noguchi.[2] Note should be taken of the greater activity in this respect of eosin "gelb" as compared with eosin "rein."

(*b*) *Eosinized Glucose Agar.*—The advantage for the study of *B. tetani* which glucose agar has over glucose bouillon is derived from the fact that in the former medium sporulation is much more abundant, and is already easily noticeable after twenty-four hours growth. At the end of seven days spores are numerous, and at the expiration of about thirty days, no vegetative bacilli remain. Hence, to glucose agar the following strengths of both eosins were added, 0.01 per cent., 0.02 per cent., 0.05 per cent., 0.1 per cent., 0.2 per cent., 0.5 per cent., and 1 per cent. Stab inoculations were made; cultivation at 37° C.

The growths were well-marked in the tubes below 0.05 per cent. strength; above this limit the growths diminished progressively with increase in concentration. On microscopical examination, it was found that 0.1 per cent. of eosin was the limit of concentration for spore formation. Below this concentration, sporulation took place; at and above it, no sporulation occurred even after three months. The number of spores in eosinized media is below the number in ordinary media; the situation of the spores in the former media is the centre of the body. Transplantation of the atypical sporulating bacilli to simple glucose agar brings about immediate

[2] *Op. cit.*

return to typical mode of sporulation. The limit of germination is for eosin " rein " about 0.5 per cent., while for complete germination, it is about 0.2 per cent. Strengths above 0.5 per cent. inhibit vegetation of spores, but they do not kill the spores. Eosin "gelb" is somewhat more inhibitory. Concentrations above 0.02 per cent. prevent sporulation, but permit vegetation; above 0.5 per cent. no vegetation occurs.

(*c*) *Eosinized Tissue Bouillon.*[3]—The tetanus bacillus grown aerobically at 37° C. in tissue bouillon produces abundant spores in a few days. The sediment which forms in the cultures in a few days, is composed largely of spores. The effect of eosin " gelb " on this process was studied by adding strengths of 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., and 2 per cent. of the dye. The tissue absorbs a part of the dye; the cultures were incubated at 37° C. for twenty hours when first examined, and subsequently for twenty-nine days longer.

After 20 Hours.		
Control.	Good growth; gas formation.	C.S.* single bacilli; no spores.
Eosin "gelb" 0.001%	Idem.	C.S. chiefly single, few threads, no spores.
" " 0.003	Idem.	C.S. bacilli chiefly in chains, no spores.
" " 0.01	Less growth.	C.S. long threads only showing vacuoles and irregular contours.
" " 0.03	Idem.	Idem.
" " 0.1	Slight growth.	C.S. very few single bacilli, small number of spores.
" " 0.3	Doubtful growth.	C.S. few bacilli and spores.
" " 1	No growth.	C.S. very few spores.
" " 2	Idem.	Idem.
After 30 Days.		
Control.	Fluid clear; deposit heavy.	C.S. all bacilli bear spores.
Eosin "gelb" 0.001%	Idem.	C.S. numerous threads and spores.
" " 0.003	Idem.	C.S. nearly exclusively in threads; very few spores.
" " 0.01	Idem.	C.S. threads; no spores.
" " 0.03	Idem.	Idem.
" " 0.1	Slight deposit.	C.S. a few short chains; many single bacilli; few spores.
" " 0.3	No change.	
" " 1	No change.	
" " 2	No change.	

* C.S. indicates cover slip preparations stained in the usual way.

[3] Theobald Smith, *Jour. of the Boston Soc. of the Med. Sciences,* 1901, iii, 340. Tarozzi, *Centralbl. f. Bakt.,* etc., Orig., 1905, xxxviii, 619.

The foregoing observations are in conformity with those already recorded. In spite of the vigorous growth of the bacilli in this medium containing small quantities of eosin, the biology of the organisms is influenced directly as regards their power to segment and to form spores. With increasing concentrations of the eosin these effects become more pronounced, until in the concentration of 0.1 per cent. only part of the transplanted spores are enabled to germinate, and the vegetative bacilli produced by this imperfect germination are restrained from free multiplication. This process of imperfect vegetation comes, I believe, to play a very interesting part in developing immunity in inoculated rats treated with eosin, of which phenomenon I will have occasion to speak hereafter.

(*d*) *Bactericidal and Sporocidal Properties of Eosin upon B. tetani.*—Glucose bouillon cultures of the tetanus bacillus do not form spores within forty-eight hours, hence such cultures can be employed to test the bactericidal effect of strong solutions of eosin. It was found that such spore-free cultures when mixed with eosin to the concentration of 2 per cent., and kept for fifteen minutes in diffuse light, fail to grow upon replantation. If the eosin strength falls below 1 per cent., not all the bacilli are killed. Contact for twenty-four hours of vegetative sporeless bacilli with eosin in solutions above 0.1 per cent., causes their death. The bactericidal effect of eosin is increased by exposure to the sun: eosin in a strength of 0.02 per cent. can cause in eight hours the death of vegetating bacilli, when exposed directly to the sun's rays. If the experiments are made under anaerobic conditions the results are not essentially different.

The spores of the tetanus bacillus display far greater resistance. Solutions of eosin of 5 per cent. and 0.05 per cent. failed to bring about their destruction after exposure under aerobic and anaerobic conditions to the direct rays of the sun for thirty hours.

(*e*) *Viability of B. tetani in Eosinized Media.*—Cultures eighty-eight days old of the tetanus bacillus in glucose agar containing 0.1 per cent. eosin "gelb" showed no spores. On transplanting from these cultures into glucose agar, a feeble growth containing few spores after seven days was secured. Transplantations into esoin-free media from this slight growth gave cultures which be-

haved, in all respects, in a normal manner. From this experiment the conclusion can be drawn that concentrations of eosin which are not quickly fatal to sporeless tetanus bacilli reduce in the first generation their power of reproduction but do not suffice to kill them outright even after long periods of contact.

(*f*) *Can B. tetanus be Rendered Durably Asporogenous by Eosin?*—Eosin in the strength of 0.01 per cent. reduces spore-formation in *B. tetani* and in the strength of 0.1 per cent. prevents it entirely. A strain of *B. tetanus* was cultivated in 0.01 per cent. and 0.1 per cent. successively through many generations. At each transfer to eosinized medium a control culture was made in glucose-agar to observe the point of final disappearance of the spore-bearing faculty. In the case of eosin "rein," the bacillus withstood fairly well the successive implantations into the eosinized medium, but the faculty to produce spores in ordinary media was not lost after eight generations, covering a period of three months, in eosinized glucose agar. A reduction of the spore-formation was noted in the first generations in the plain glucose medium. In the case of eosin "gelb," the bacillus survived for three generations only in 0.1 per cent. eosin-glucose-agar, and for the eight generations in the 0.01 per cent. medium. No permanent alteration of spore-bearing capacity was effected in the latter cultures.

(*g*) *Is the Toxin Producing Power of B. tetani Affected by its Growth in Eosinized Media?*—Two cultures of *B. tetani* were kept in 0.1 per cent. eosin "gelb" glucose-agar for eighty-eight days, after which they were renewed by transplantation to glucose bouillon. After forty-eight hours growth at 37° C., these cultures and suitable control cultures were filtered through porcelain. The toxicity of the filtrates was approximately equal. The deduction from this experiment is obvious: no permanent influence upon the toxin-producing faculty of *B. tetani* is exerted by long contact of eosin in concentrations below the bactericidal limit.

THE ACTION OF EOSIN UPON THE TETANUS BACILLUS IN VIVO.

The next subject of the study taken up related to the manner in which eosin acts in preventing tetanus in rats inoculated with tetanus bacilli or their spores.[4] The effects to be explained are,

[4] Flexner and Noguchi, *op. cit.*

briefly, these: rats, beneath the skin of which tetanus spore-threads are placed, regularly develop tetanus and die. If, however, the inoculated rats are treated by injections of eosin about the spore-thread, many recover, and some even fail to develop any symptoms of tetanus. Injection of the eosin in other parts of the body may delay the appearance of tetanus and the fatal issue, but does not suffice wholly to prevent them. The effect of the eosin on the local reaction to the tetanus bacilli in the inoculated rats is to be explained.

Fate of Spores Introduced into the Body on Threads.—Eighteen rats (weighing about 90 grams each) were inoculated beneath the skin of the thigh with spore-threads of *B. tetani,* free from toxin, on June 8, 1906. The six of these left untreated (controls) developed tetanus in two to six days time (average, three days). Death usually resulted on the third day after the appearance of the tetanus. Cultures were made from the threads and the liver.[5] Tetanus bacilli were recovered from the threads in all six animals and from the liver in three animals. The remaining twelve rats were treated with eosin "rein." Doses of 0.2 to 0.4 c.c. of a 2 per cent. solution were injected successively on three days, beginning immediately after the inoculation, about the thread, and intermittently (every other day) for three more injections. The treated animals did not develop tetanus, and a part of them were alive and healthy four months after the inoculation.

The first question which I asked myself is, *Do the tetanus spores germinate and multiply under the influence of the eosin, and what becomes of the germinated bacilli?*

Experiment. Rat No. 7. A part of the spore-thread was removed forty-eight hours after inoculation and during the eosin treatment. Cover slips prepared from it showed many spores, a small number of bacilli and a few leucocytes. A second portion of the thread was removed on the ninth day (day of last eosin injection). Cover slips showed very few bacilli and many spores and leucocytes. Cultures gave pure growths of *B. tetani.* On the thirteenth day, the remainder of the thread was removed. Now only spores could be found, and leucocytes were no longer present. The rat remained well.

Rat No. 8. Same treatment; examination on fifth day; among many spores a small number of bacilli—vegetative forms—were seen.

[5] Tarozzi, *Centralbl. f. Bakt.,* etc., 1906, xl, 305, 451.

The study of the spore threads in the two inoculated rats show conclusively that the injections of eosin do not wholly prevent the germination of the spores, but that the germination is largely suppressed; and it also renders the view very probable that of the germinated spores few or none multiply in the eosinized tissues, while after a short time (few days) the vegetated bacilli themselves disappear. Attention is called to the observation that the tetanus spores remain in an intact state for many days in the inoculated region of the body.

The next question which I asked myself is, *What becomes eventually of the ungerminated spores, and, supposing they remain locally in the tissue, do they suffer any alteration in toxin-producing power?*

Experiment. Rat No. 9. Inoculated June 8. Eosin-treated. No symptoms, June 24. Removed the spore-thread which was found to be surrounded by fibrous tissue and adherent to the fascia. Eosin had disappeared. Cover slips showed many spores and no bacilli. A pure culture of *B. tetani* obtained in tissue bouillon.

Rat No. 10. The thread was removed nineteen days after inoculation. The result was in all respects like that of rat No. 9. Toxin of the usual strength was yielded by the cultures.

Rat No. 11. A part of the spore-thread was removed twenty-three days after the inoculation. Cover slips showed many spores which yielded a pure culture of *B. tetani* containing a strong toxin. The second wound healed without giving rise to tetanus. Animal remained well.

By these observations it is shown that the healing of the local wound containing the eosinized threads, proceeds in a manner similar to that of a sterile wound; and it is also shown that, by virtue of the eosin treatment, the tetanus spores are reduced to the value of inocuous foreign bodies. In the course of this process, the tetanus spores, already quiescent, remain in the healed tissues for an indefinite time. They are not devitalized by the tissues, or, apparently, altered in any essential way, at least, their toxin-producing power is not impaired by the new conditions under which they survive.

If the results obtained in vitro and in vivo are compared certain correspondences, as well as certain differences, can be noted. Perhaps the most important difference consists in the disappearance of the vegetative bacilli in the body, and their persistence in the test tubes, in the tests with eosin; and the germination of the re-

strained spores in the culture once the eosin is no longer present, while, in the body, the tissues already advanced in healing restrain the vegetative propensities of the spores, although the toxin has been entirely eliminated.

The healing of the wound of the second operation in rat No. 11, without giving rise to tetanus, all the eosin having meanwhile disappeared, is an interesting fact, and one that deserves further consideration. The cultivation experiment proved that living tetanus spores, capable of toxin-production remained in the thread; and yet the remainder of the thread, in the tissues, gave rise to no symptoms. It might be thought indeed, that the spore threads, after this long sojourn in the body, are not capable of directly infecting a second animal. A test of this possibility was easily made.

Experiment. Rat No. 12. A normal rat was inoculated on July 9 beneath the skin of the thigh with a spore-thread removed on the thirty-second day from an eosin-treated rat. On July 11 the animal was tetanic, and on July 13 it was dead. A pure culture of *B. tetani* was recovered from the thread.

The apparent innocuousness of the second operation for the removal of spore-threads in animals treated long before with eosin, and the great susceptibility of the normal rat to the threads after this long residence in the body, brought up the question of the possible existence of a local immunity to tetanus in the eosin-treated animals. This idea was, of course, capable of experimental verification. The experiments relating to it form the subject of a separate publication.

SUMMARY.

Eosin, if present in cultures containing tetanus spores, prevents the germination of these spores when its concentration (in glucose bouillon) reaches 0.2 per cent. When the concentration of the eosin sinks to 0.01 per cent., germination of the spores is no longer inhibited, but the vegetative bacilli developed from the spores execute a highly restrained form of multiplication. When the eosin concentration sinks to 0.001 per cent., vegetation and multiplication of the bacilli become more active, but no new spores are formed even after long periods of time. With glucose agar it is not until the concentration of the eosin in the cultures falls to 0.05 per cent. that sporulation again appears. At this concentration of the eosin,

very few spores are formed; but as the eosin sinks lower and lower, sporulation becomes more active, until with 0.001 per cent. it is essentially of normal degree. In concentrations of 0.003 per cent., eosin prevents perfect segmentation of the multiplying bacilli, with the result that, finally, long and convoluted threads of bacilli are produced. The spores which are formed in a medium containing 0.01 per cent. of eosin are often situated at the centre and not at one pole of the bacilli.

Eosin in a strength of 2 per cent. is capable of destroying the vegetative bacilli, if the contact is prolonged to fifteen minutes, and in strength of 0.1 per cent., in twenty-four hours. Placing this latter mixture of bacilli and eosin in the sunlight greatly hastens the bactericidal effect, and the bacilli are found to be incapable of growth at the end of several hours. Eosin in high concentrations is not capable of killing the tetanus spores, even after long exposure to sunlight (thirty hours).

The toxin production of tetanus bacilli grown in eosinized culture media diminishes as the concentration of the eosin increases. This effect is brought about partly by the restraining action of the dye on vegetation, and partly by its detoxicating action upon the poison.,

The toxin-producing power and the virulence of tetanus bacilli are not permanently modified by contact with eosin for a long period, or by successive cultivations in eosinized media.

Eosin is likewise capable of restraining the vegetation of tetanus spores in the animal body. In spore threads inserted beneath the skin of rats, and surrounded with eosin in solution, a very restricted vegetation takes place. If the injections of eosin are repeated, vegetation soon ceases and the vegetated bacilli degenerate and disappear.

The ungerminated tetanus spores remain alive in a latent condition indefinitely in the healed wound beneath the skin. These spores do not lose power to grow outside the body, or inside the body of animals under favorable conditions, or to produce toxin in a characteristic manner.