CLXV. OXIDATION-REDUCTION STUDIES IN RELATION TO BACTERIAL GROWTH.

III. THE POSITIVE LIMIT OF OXIDATION-REDUCTION POTENTIAL REQUIRED FOR THE GERMINATION OF *B. TETANI* SPORES *IN VITRO*.

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The determination of the most positive level of oxidation-reduction (O/R) potential at which spores of *B. tetani* can germinate has been previously attempted by one of us [Fildes, 1929]. Clark's O/R dyes were used to measure the potential of the culture media to which the spores were added. With the technique then available there was great difficulty, firstly in determining the degree of reduction of the dyes, and secondly in maintaining a constant potential level while the spores were "trying to germinate." Poising the O/R potential by hydrogen-air mixtures was not completely successful owing to the lack of a method for controlling the composition of the gas mixture and for relating this to the potential of the medium. Thus it was possible for the medium to attain an unnecessarily negative potential before the spores had had time to overcome any lag due to causes other than O/R potential. The most positive limit for germination found might therefore be too negative.

In two previous papers [Knight, 1930, 1, 2] a convenient electrometric technique for observing and poising the O/R potential of culture media was described. The present communication records the results of the application of this method to the determination of the positive O/R potential limit for the germination of spores of *B. tetani*.

Experimental.

The method used was to poise the O/R potential of a sample of sterile culture medium at a constant level by passage of a stream of very dilute oxygen in nitrogen, controlled by simultaneous electrometric observation of the potential. In some experiments Clark's O/R dyes were used in the media to facilitate the poising [Knight, 1930, 2]. When the potential had been

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observed to remain constant, spores were added to the poised medium in the electrode vessel. Potential readings were made about every hour.

Samples of the inoculated medium were taken with a sterile capillary pipette. If a little air was expelled from the pipette before introduction into the culture medium the potential was not altered. The sample was centrifuged in a very small tube, and a film was prepared from the (invisible) sediment. The film was stained in the usual way for spores with carbolfuchsin and methylene blue. By taking samples at intervals the onset of germination

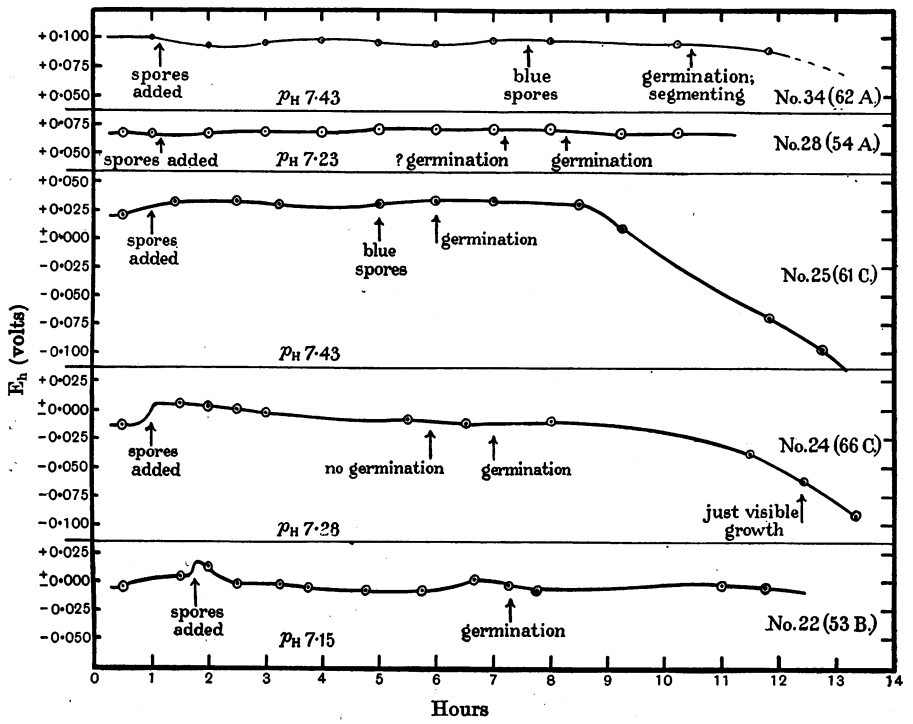

Fig. 1. Determination of time of germination of *B. tetani* spores in buffer-broth at constant E_h level.

could be detected. The preparation of the spore suspension used for inoculation and the recognition of germination have been described by Fildes [1929]. The medium used throughout the experiments was peptone-beef infusion buffered with KH_2PO_4 or KH_2PO_4-asparagine ($M/20$). The reaction was adjusted approximately to the required p_H from a hydrogen electrode titration curve. After autoclaving, the exact p_H of every sample was determined by the hydrogen electrode. The strain of *B. tetani* used was "TM. 8," the optimum p_H for this strain, tested at low E_h levels, being 7·1–7·9, the acid limit for germination p_H 6·0, the alkaline limit p_H 8·5.

Table I.

No.	Exp.	E_h (±0·005 v.)	p_H	Time of germination (hours): No germination	Time of germination (hours): Germination observed	Remarks
				A. p_H 6·4 to 6·9.		
2	65A	+0·01	6·87	6·5 (blue spores)	8	Just germinated
3	53A	+0·03	6·40	10	24	Germination time <24 hours
4	66A	+0·04	6·53	10·25	11·5	Just germinated
5	66B	+0·05	6·70	—	10·25	,,
6	44B	+0·06	6·47	9	19	Heavy growth in 19 hours
7	52A	+0·10	6·53	13	24	Dye present
8	52B	+0·105	6·53	20	24·5	No dye. Less advanced than 52A
9	59A	+0·11 to +0·14	6·86	28	—	—
10	58B	+0·125	6·78	12·5	24	Germination time <24 hours
11	58A	+0·12 to +0·14	6·74	12·5	24	,, ,,
				B. p_H 7·0 to 7·1.		
12	65B	+0·01	7·10	5 (blue spores)	6	Just germinated
13	61A	+0·065	7·10	7·5	11	,,
14	54C	+0·08	7·03	9 (blue spores)	10	—
15	56A	+0·095	7·05	8	12	—
16	57A	+0·10	7·05	11·5	23	—
17	59B	+0·105	7·08	12	24	No continued growth in 5 hrs.
18	62C	+0·11	7·10	9·5	11	Just germinated
19	63A	+0·13	7·10	35	—	E_h reduced to +0·04 in 4 hrs. → blue spores
				C. p_H 7·15 to 7·65.		
20	36A	−0·05	7·16	3	4	Just germinated
21	49A	±0·00	7·23	4·5	5	,,
22	53B	±0·00	7·15	—	5·5	,,
23	53C	±0·00	7·18	—	6	,,
24	66C	±0·00	7·28	4·75	6	,,
25	61C	+0·03	7·43	4	5	,,
26	56C	+0·065	7·54	8 (blue spores)	12	Did not continue to grow
27	50B	+0·07	7·16	6	(8)	Heavy growth in 24 hrs.
28	54A	+0·07	7·23	6 (blue spores)	7	Just germinated
29	58C	+0·07	7·64	5	7	,,
30	55A	+0·072	7·50	8	11	,,
31	62B	+0·08	7·28	7·5 (blue spores)	10·25	Germination time <10 hrs.
32	55B	+0·08	7·50	8	11	Just germinated
33	59C	+0·095	7·41	6	12	Beginning to segment
34	62A	+0·095	7·43	7·5 (blue spores)	10·25	Germination time <10 hrs.
35	56B	+0·105	7·18	—	8	Just germinated
36	63C	+0·112	7·43	35	—	E_h reduced to +0·005 in 4 hrs. → germination
37	48	+0·12	7·31	24	—	Growth in 1 hr. after $Na_2S_2O_4$
38	63B	+0·125	7·28	35	—	E_h reduced to +0·03 in 4 hrs. → germination
39	61B	+0·135	7·28	24	—	No germination
40	50A	+0·135	7·20	63	—	Toluylene blue; spores not viable
41	49B	+0·145 to +0·135	—	18	—	No germination
			7·22	—	30–40	? Germination: no continued growth
42	47	+0·165	7·30	48	—	No germination

Results.

E_h-time curves. Typical *E_h-time* curves showing the determination of time of germination at various levels of O/R potential are given in Fig. 1. The results of these and other similar experiments are collected in Table I. In the figures "blue spores" signifies the observation of the first phase of germination, and "germination" signifies the observation of a bacillus completely emerged from the ruptured spore case ("hatched"). In some experiments O/R dyes were present in the culture media. In otherwise similar experi-

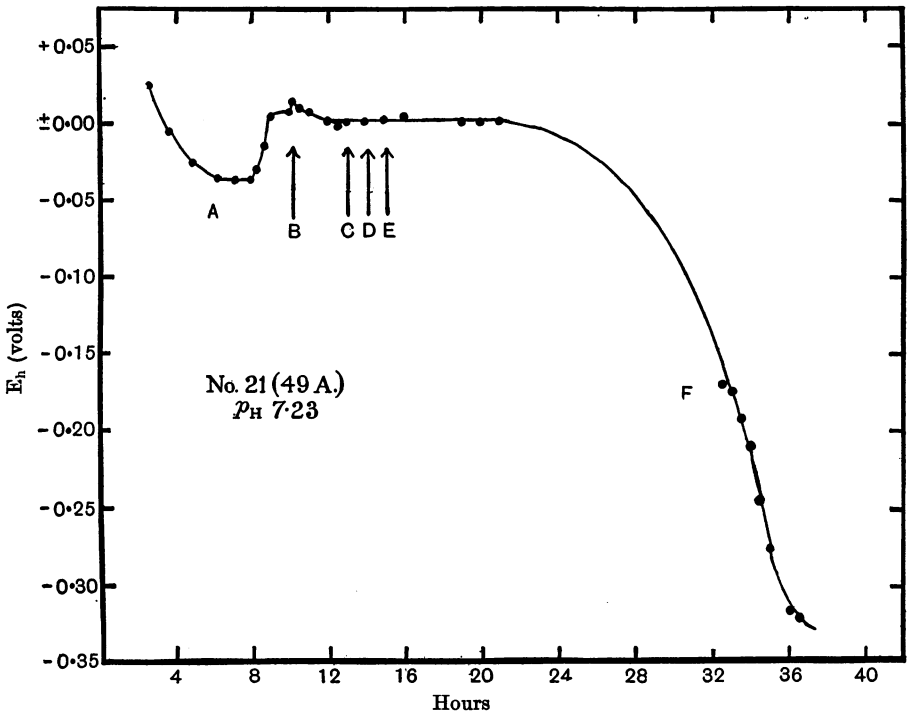

Fig. 2. Complete germination and growth curve of *B. tetani*.

A. By-pass manipulation to poise E_h at ± 0·00 volt.
B. Addition of *B. tetani* spores.
C. No germination 3 hours.
D. ? Germination 4 hours.
E. Germination observed 5 hours.
F. Visible growth.

ments, no significant differences in the times of germination with and without dyes were observed.

The curves show that the normal fluctuation during an experiment was about 0·005 v. The reaction of the medium was usually adjusted between p_H 7·15 and 7·65 (within the optimum range for the micro-organism), so that the effect of O/R potential level alone could be observed.

On addition of the inoculum there was little or no change of potential. When poised at fairly negative levels there was a slight temporary positive shift (0·01–0·015 v.) due to the small amount of oxygen dissolved in the spore

suspension. This could be completely avoided by de-aerating the suspension before inoculating. The spores as such (in the concentrations used in these experiments) did not affect the potential. This was constant until after germination, when the bacilli were actively proliferating (Fig. 1, Nos. 25 (61 c), 24 (66 c), and Fig. 2).

The complete curve for one experiment (Fig. 2) shows the poising of the O/R potential and the relative effects on the potential of inoculation, germination, and proliferation. Generally the experiment was stopped after germination had been observed and segmentation had begun. We were only concerned here with the conditions at the *initiation* of growth.

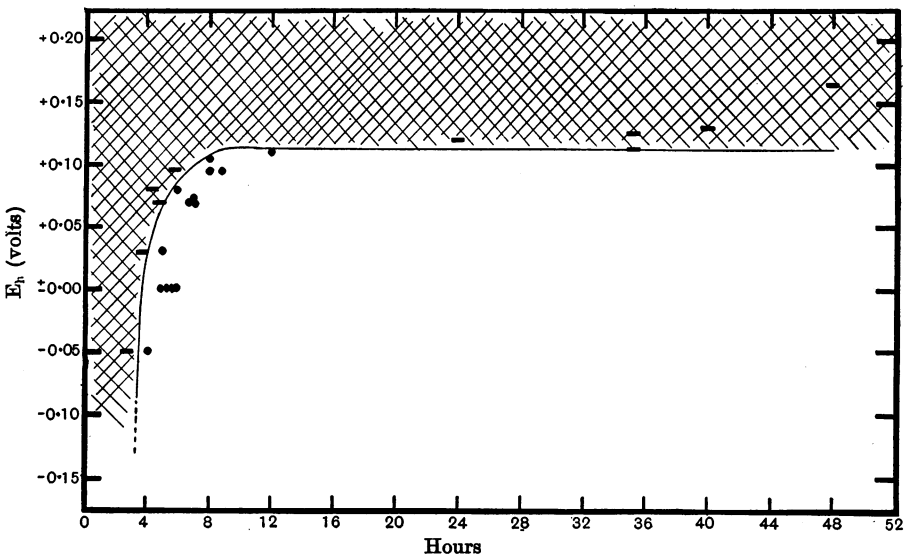

Fig. 3. Variation of time of germination of *B. tetani* spores with E_h level. p_H 7·15–7·65.

– No germination observed. • Germination first observed.

Relation between O/R potential and time of germination. The data of Table I c are plotted in Fig. 3, which shows the relation between time of germination and O/R potential level. The shaded area shows the field where the spores had not germinated. When E_h was negative to $-0{\cdot}05$ v., the shortest time after the addition of spores in which germination was observed was 4 hours. At progressively more positive levels the time lag increased until at $E_h + 0{\cdot}10$ v. it was 8–10 hours. At the same time each phase of germination became lengthened. Blue-staining spores were then first observed considerably after the normal time of "hatching" at lower levels (Fig. 1, No. 34 (62 A)). When finally "hatched" the bacilli were often malformed and did not stain well, and segmentation was much delayed. In no case did germination and continued growth take place when the potential was more positive than

E_h + 0·011 v. within the range p_H 7·0–7·65. Two experiments (Table I A, Nos. 10 and 11) indicated that a slightly more positive E_h (+ 0·12–0·13) could be tolerated if the reaction was slightly more acid, viz. p_H 6·75.

Viability.

In a number of experiments (Table I C, Nos. 36 to 42) the potential was maintained positive to E_h + 0·11 v. for 24 hours or more without germination. The spores were still viable however and germinated rapidly on lowering the potential. Thus in Exp. 48 (Table I C, No. 37) there was no germination in 24 hours at E_h 0·12 v. (p_H 7·31). On addition of $Na_2S_2O_4$ which produced a potential of the order of E_h − 0·53 v. germination was observed in 1 hour instead of the usual minimum of 4 hours. Evidently part of the lag, in experiments timed from the addition of the spores to the medium, is due to acclimatisation of the spore to nutritional and other factors of the environment, apart from O/R potential. In one experiment with boiled toluylene blue present (the dye was decomposed by the boiling) spores were found to be non-viable, after they had been maintained at E_h + 0·14 v. (p_H 7·2) for 60 hours. No other case of toxicity of a dye was noticed.

Effect of p_H on lag.

If the p_H of the medium was outside the optimal range, the time of germination was increased as expected. The effect of unfavourable p_H is then combined with the effect of O/R potential. Thus at E_h + 0·03 v. the germination time was 5 hours at p_H 7·43, but more than 10 hours at p_H 6·4 (Table I, Nos. 25 and 3).

Discussion.

The general conclusion from these experiments is that spores of *B. tetani* will not germinate at all, in an otherwise favourable medium, when the O/R potential is more positive than E_h + 0·11 v. (p_H 7·0–7·65). This confirms in general the results obtained by Fildes [1929], namely, that there is a positive limit of O/R potential beyond which germination will not take place. The value of the limiting potential found in the present work is 0·10 v. more positive than that found by Fildes. This was anticipated, as Fildes failed to poise his medium at the high levels (positive to E_h + 0·05 v.) long enough to observe germination at those levels.

Fildes's level (about E_h ± 0·00 v. at p_H 7·2) may be taken to be the positive limit at which germination will take place readily. In the zone between this level and that found in the present work germination is inhibited to a greater or less degree by inappropriate conditions of O/R potential. Thus in a narrow zone just negative to the completely inhibitory level (E_h + 0·11 v.) a few bacilli may emerge without the usual intense blue-staining reaction and thereafter entirely fail to segment and grow. It would appear that conditions may be just adequate for the phase of germination which takes place within the spore case, but inadequate for continued life outside the spore. Conceivably

the potential measured in the external medium is more positive than that within the spore case.

The present experiments were not designed to decide whether there is any specific "toxic" effect of oxygen distinct from its action in maintaining a positive potential. Whatever the mechanism of anaerobic growth may be, it is evident that there is a definite correlation between the ability of spores to germinate and the O/R potential of the medium as measured by an "inert" electrode immersed in it.

SUMMARY.

The effect of O/R potential on the germination of spores of *B. tetani* has been studied by an electrometric method. The spores were subjected to different potential levels, which were maintained constant during the course of the experiment. Germination was completely inhibited at potentials more positive than $E_h + 0{\cdot}11$ v. (p_H 7·0–7·65).

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REFERENCES.

Fildes (1929). *Brit. J. Exp. Path.* **10**, 151.
Knight (1930, 1). *Biochem. J.* **24**, 1066.
—— (1930, 2). *Biochem. J.* **24**, 1075.