The Germination of Spores of *Clostridium tetani*

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SUMMARY

The spores of *Clostridium tetani* will germinate aerobically and anaerobically in complex media such as heart infusion broth or tryptone with yeast extract at 37° and pH 7.5. A synthetic medium as effective as heart infusion broth for germination under both aerobic and anaerobic conditions consists of 1 mM-methionine, 100 mM-lactate, 82 µM-nicotinamide, 280 mM-Na⁺ in 40 mM-phosphate buffer, pH 7.5. Anaerobic germination would take place in either methionine with Na⁺ or lactate and nicotinamide with Na⁺, but all the germinants were essential aerobically. Heat activation did not occur and thioglycollate increased the rate of germination.

INTRODUCTION

Most of the work on the germination of the bacterial spore has been directed towards the aerobic sporing bacilli (see Gould & Hurst, 1969). With clostridia the germination requirements are often complex (Hitzman, Halvorson & Ukita, 1957; Gibbs, 1964, 1967; Fujioka & Frank, 1966; Bergère, 1969; Holland, Barker & Wolf, 1970; Rowley & Feeherry, 1970; Barker, Gould & Wolf, 1971).

The present study was undertaken to examine the early stages of development from the spores of *Clostridium tetani* with the object of finding an explanation for the unexpected toxicity of heat- and radiation-damaged spores of this organism (Shoesmith, 1964). Fildes and his colleagues established the oxidation-reduction potential necessary for the germination and outgrowth of *C. tetani* (Knight & Fildes, 1930) and Kaufman & Humphries (1958) described a synthetic medium suitable for the vegetative growth but the necessary nutritional factors for germination were not known. Our preliminary tests showed that simple meat infusions and tryptic or peptic digests had a poor capacity to stimulate germination, even though they would support vegetative growth. In media of a more complex nature, such as heart infusion broth (Difco), most of the spores germinated within 1 h (J. G. Shoesmith, unpublished observations). These results provided a basis for the determination of the substances stimulatory for germination in *C. tetani* and a preliminary report has already been made (Shoesmith & Holland, 1968).

METHODS

Organism. *Clostridium tetani* CN761 (Wellcome Research Laboratories Culture Collection) was used throughout the work and was maintained in cooked meat medium (Southern Group Laboratory, Hither Green Hospital, London, S.E. 13).

Production and harvesting of spores. Cooked meat medium, (80 ml), after centrifuging at 2500g for 30 min to remove meat particles, was held in polypropylene centrifuge tubes (MSE, Crawley, Sussex) and inoculated with 1 ml of an 18 h culture of the organism and incubated at 37° in an anaerobic jar. After 5 days, 60 to 70 % of the organisms in the culture

were phase-bright spores and about 8 % were phase-dark spores. The organisms were washed in distilled water by centrifuging, the first three times at 2500g for 30 min and then repeatedly at 430g for 30 min until over 90 % of the organisms were phase-bright spores and no vegetative cells were present. All centrifugings were carried out at 5° to minimize spore germination and the pellets were dispersed with sterile glass beads (4.5 to 5.5 mm diam.). The final spore suspensions were stored at 4°. These spores were always contained in their sporangia. Since the sporangia could not be removed without enzyme treatment most experiments were carried out with the sporangia present.

Media and chemicals. The following media were prepared in stock aqueous solutions at a sufficiently high concentration to allow dilution and addition of other components to give appropriate final concentrations; they were sterilized by autoclaving at 15 lb/in² for 15 min: heart infusion broth (Difco), 10 % (w/v); Tryptone T (Oxoid), 20 % (w/v); yeast extract (Difco), 8 % (w/v).

Amino acids, purines, pyrimidines, vitamins and coenzymes were prepared in stock aqueous solutions at 200 mM in most cases and stored frozen at -20° ready for dilution to the required final concentration. Thioglycollic acid and ascorbic acid were prepared at 200 mM at pH 6.8, stored at 4°, and diluted to a final concentration of 10 mM. NaCl (spectroscopic grade) 2 M and NaH₂PO₄ - Na₂HPO₄ buffer, 200 mM, pH 7.5, were stored at 4° and the latter used at 40 mM.

Measurement of germination. Germination was most conveniently determined by phase-contrast microscopy of slide-culture preparations. Mixtures of compounds to be tested were prepared from stocks in suitable proportions and the pH adjusted with M-HCl or M-NaOH, the volume made up to 5.5 ml and kept at -20° until needed. The desired Na⁺ concentration was obtained by addition of NaCl. Before testing, the media were thawed and 0.55 ml was added to 100 × 12 mm tubes at 50° containing 0.05 ml sodium thioglycollate, 200 mM, pH 6.8 to maintain anaerobic conditions and 0.4 ml 2 % Oxoid no. 2 agar, pH 7.5, previously melted and brought to 50°. For aerobic conditions water replaced the thioglycollate. A loopful of spore suspension in distilled water was allowed to dry on a no. 1½ coverglass at room temperature and inverted over three drops of the medium sufficient to fill the well of a 1 × 3 in hollow-ground slide warmed to 50°. The slides were incubated at 37° in a covered tray (6.5 × 7 × 2.5 in) immersed almost to the lid in a water bath. The slides were removed at intervals to obtain counts of phase-bright spores and of the total number of spores using a Beck (Ealing-Beck Ltd, Greycaine Road, Watford, WD2 4PW) binocular phase-contrast microscope fitted with a mechanical stage. The spores were examined by means of a 2 mm oil-immersion phase-contrast objective and × 10 eye-pieces with green-filtered illumination from a high-intensity lamp. A graticule in one eye-piece facilitated counting and the numbers of phase-bright and total spores were recorded on tally-counters. The counting of 100 spores thus gave a direct count of the percentage of phase-bright spores.

In a few preliminary experiments the effect of germinants was determined from the extinction at 600 nm. To do this it was necessary to obtain spores free from their sporangia and this was accomplished by treatment with an enzyme system produced by a mould (J. G. Shoesmith, unpublished observations).

Unless otherwise stated all germination tests were carried out in the presence of 10 mM-sodium thioglycollate to maintain anaerobic conditions. Heart infusion broth (2.5 %, w/v) and 10 mM-thioglycollate were used as positive and negative controls.

Table 1. *The effect of heating on the subsequent germination of spores in 2.5 % heart infusion broth anaerobically at 37°, pH 7.5*

Heating for 10 min at	Time for 50 % germination (min)	Viable count ($\times 10^{-6}$)
4°	21	—
70°	21	40
75°	21	43
80°	21	45
85°	24	35
90°	32	33
95°	54	34
100°	420	5.0

Table 2. *Anaerobic germination in complex media*

Medium	Phase-bright spores after 2 h at 37° pH 7.5 (%)
2.5 % heart infusion broth (HIB)	4
2.5 % HIB diffusible fraction*	5
2.5 % HIB non-diffusible fraction*	85
2.5 % HIB non-diffusible and diffusible fractions recombined	5
5.0 % Tryptone T	52
2.5 % Tryptone T	66
2.0 % yeast extract	86
1.0 % yeast extract	83
5 % Tryptone T + 1 % yeast extract	30
5 % Tryptone T + 1 % yeast extract + 10 mM-NaHCO ₃	14
Control, no germinants	91

* Heart infusion broth was dialysed against distilled water at 4° and the diffusible and non-diffusible fractions freeze-dried and redissolved in water to the original volume.

RESULTS

The effect of heating on spore germination

There was no change in the viable count and rate of germination of the spores of *Clostridium tetani* after heating at temperatures in the range 70° to 85° (Table 1). Complete germination occurred in both heated and unheated spores. Heating at higher temperatures decreased the rate of germination. There was therefore no evidence of heat-activation with these spores and they were not heated in the following experiments concerned with the determination of the germinants.

Determination of germinants

Preliminary experiments using changes in extinction to detect germination showed that 2.5 % (w/v) heart infusion broth could be replaced by 2.5 % (w/v) Tryptone T combined with 1.0 % (w/v) yeast extract. This method required the spores to be freed from their sporangia, and also changes in extinction did not always correspond to changes in phase-contrast appearance, ability to take up simple stains and heat resistance. Estimation of the proportion of phase-bright spores was therefore used to measure germination in all other experiments and results obtained by this method showed that the necessary factors for

Table 3. *Effect of vitamins on anaerobic germination of spores*

Medium*	Phase-bright spores after 2 h at 37°, pH 7.5 (%)
2.5 % heart infusion broth	4
5 % Tryptone T + 1 % yeast extract + 10 mM- NaHCO ₃	15
5 % Tryptone T + 10 mM-NaHCO ₃ (GM 1)	23
GM 1 + all vitamins† given below	6
GM 1 + nicotinamide	7
GM 1 + NAD	12
GM 1 + NADP	13
GM 1 + biotin	19
GM 1 + <i>p</i> -aminobenzoic acid	27
GM 1 + calcium pantothenate	24
GM 1 + thiamine hydrochloride	22
GM 1 + pyridoxin hydrochloride	28
GM 1 + folic acid	25
GM 1 + riboflavin	23
Control, no germinants	91

* 10 mM-thioglycollate was present in all cases.

† Vitamins were incorporated at 10 µg/ml.

Table 4. *Effect of carboxylic acids on the anaerobic germination of spores in the presence of amino acids*

Medium	Phase-bright spores after 2 h at 37° (%)
2.5 % heart infusion broth	2
GM 2*	60
GM 2 + all acids given below	12
GM 2 + lactate	9
GM 2 + pyruvate	30
GM 2 + oxaloacetate	47
GM 2 + malate	50
GM 2 + fumarate	58
GM 2 + succinate	60
Control, no germinants	88

* GM 2 consists of 23 amino acids each at 1 mM (listed in Table 5), phosphate buffer (40 mM, pH 7.5), NaCl (50 mM), NaHCO₃ (10 mM), nicotinamide (82 µM).

germination were water-soluble, dialysable and could be provided with a mixture of yeast extract, Tryptone T and NaHCO₃ (Table 2).

A mixture of ten vitamins, each at 10 µg/ml, was an adequate substitute for yeast extract in this complex medium, with nicotinamide being the simplest and most effective single vitamin (Table 3). Nicotinamide therefore replaced yeast extract in further tests which showed that a mixture of amino acids and carboxylic acids with 40 mM-phosphate buffer and 50 mM-NaCl could replace Tryptone T. (The salt concentrations were chosen to simulate those in Tryptone T (Oxoid Manual, 1965).) Lactate was the most effective carboxylic acid though pyruvate had some activity (Table 4), and of the amino acids, methionine, phenylalanine and leucine were the most effective, with isoleucine being less so and alanine being somewhat inhibitory (Table 5). Germination could therefore take place anaerobically at a rate similar to that in 2.5 % (w/v) heart infusion broth in a defined medium consisting of

Table 5. *Effect of amino acids on anaerobic spore germination*

Medium	Phase-bright spores after 2 h at 37° (%)
2.5 % heart infusion broth	4
GM 3*	67
GM 3 + amino acids of group A	19
GM 3 + amino acids of group B	48
GM 3 + amino acids of group C	14
GM 3 + amino acids of groups A and B	17
GM 3 + amino acids of groups A and C	20
GM 3 + amino acids of groups B and C	17
GM 3 + amino acids of groups A, B and C	9
Group A	
GM 3 + alanine	87
GM 3 + arginine	58
GM 3 + asparagine	69
GM 3 + aspartic acid	63
GM 3 + glutamic acid	68
GM 3 + phenylalanine	29
GM 3 + leucine	15
Group B	
GM 3 + creatinine	66
GM 3 + creatine	60
GM 3 + cystine	69
GM 3 + diaminopimelic acid	67
GM 3 + glycine	68
GM 3 + histidine	61
GM 3 + hydroxyproline	69
GM 3 + isoleucine	40
Group C	
GM 3 + lysine	80
GM 3 + methionine	20
GM 3 + proline	62
GM 3 + serine	61
GM 3 + threonine	65
GM 3 + tryptophan	57
GM 3 + tyrosine	60
GM 3 + valine	71
GM 3 + methionine + phenylalanine + leucine	6
GM 3 + amino acids of groups A, B and C except methionine, phenylalanine and leucine	50

* GM 3 consists of phosphate buffer (40 mM, pH 7.5), NaCl (50 mM), NaHCO₃ (10 mM), lactate (1 mM) and nicotinamide (82 μ M).

leucine, methionine, phenylalanine, and lactate each at 1 mM, nicotinamide (82 μ M), NaHCO₃ (10 mM), NaCl (50 mM) and sodium phosphate buffer (40 mM, pH 7.5).

Testing different combinations of the various constituents showed that methionine, lactate, nicotinamide and phosphate buffer were effective, though leucine, phenylalanine, lactate, nicotinamide, NaHCO₃ and NaCl had about the same activity (Table 6). Since the former system was simpler it was chosen for more detailed examination.

Increasing the lactate concentration to 100 mM and total Na⁺ to 280 mM with 1 mM-methionine and 82 μ M-nicotinamide gave a medium capable of germinating the spores as rapidly as 2.5 % (w/v) heart infusion broth, both aerobically and anaerobically (Fig. 1). Each germinant in this medium was then omitted or varied by tenfold changes in concentra-

Fig. 1. Germination of spores of *Clostridium tetani* in 2.5% (w/v) heart infusion broth (●) and in methionine (1 mM), nicotinamide (82 μ M), lactate (100 mM) and Na⁺ (280 mM) (■) at 37°, pH 7.5. Germination under aerobic conditions is shown by the broken line, under anaerobic conditions by the solid line.

Table 6. *Anaerobic germination of spores in various synthetic media supplemented with amino acids at 37°, pH 7.5*

Compounds for basal medium	Compounds present in basal medium						
	NaHCO ₃ (10 mM)	NaCl (50 mM)	Phosphate buffer (40 mM)	Nicotinamide (82 μ M)	Lactate (1 mM)		
NaHCO ₃ (10 mM)	+	.	.	.	+	+	+
NaCl (50 mM)	+	+	.	.	+	+	+
Phosphate buffer (40 mM)	+	+	+	.	+	+	.
Nicotinamide (82 μ M)	+	+	+	+	.	+	+
Lactate (1 mM)	+	+	+	+	+	.	+
Amino acids (1 mM) included in medium;	Germination (phase-bright spores after 2 h) (%)						
	Phenylalanine	Leucine	Methionine	Phenylalanine, leucine	Phenylalanine, methionine	Leucine, methionine	Leucine, methionine, phenylalanine
Phenylalanine	35	52	75	80	62	78	25
Leucine	25	28	53	82	65	54	59
Methionine	25	7	6	78	7	39	44
Phenylalanine, leucine	7	17	47	79	73	60	16
Phenylalanine, methionine	9	9	5	76	36	37	8
Leucine, methionine	10	9	16	78	25	39	4
Leucine, methionine, phenylalanine	5	7	8	79	28	51	6

tion with the other three components constant. The pH was adjusted and maintained with tris-HCl buffer for tests on the effect of Na⁺. Lactate was required in higher concentration than methionine or nicotinamide and Na⁺ appeared essential (Fig. 2*a*). Chloride ions were not involved since tris-HCl buffer or KCl did not stimulate germination in the absence of NaCl. Parallel experiments using aerobic conditions (Fig. 2*b*) confirmed the necessity for Na⁺ and also showed that under these conditions all the components were essential.

Variation of one or more germinants indicated that there may be two germination systems. Under anaerobic conditions germination will take place in either methionine and Na⁺ or lactate, nicotinamide and Na⁺, though all the compounds are necessary for the most rapid germination (Table 7). Some earlier results (Shoesmith & Holland, 1968) suggested that a

Fig. 2. The rate of germination of *Clostridium tetani* under anaerobic (a) and aerobic (b) conditions with concentrations of germinants varied one at a time (see text). Times for 50% germination were derived from graphs of percentage germination against time. The symbols with arrows indicate that the 50% germination time was > 1000 min. ■, Nicotinamide; ▲, methionine; ●, lactate; ▼, Na⁺.

Table 7. Anaerobic germination of spores in media of different composition

Compounds for basal medium*	Compounds present in basal medium						
	+	+	+	.	.	.	+
L-Methionine (1 mM)	+	.	+	+	.	+	.
Sodium lactate (100 mM)	+	+	.	+	+	.	.
Nicotinamide (82 µM)	+	+	.	+	+	.	.
	Germination						
Phase-bright spores at 3 h (%)	2	21	12	12	98	97	18
Phase-bright spores at 6 h (%)	0	12	3	3	96	93	7

* Basal medium also contained 280 mM-Na⁺ and 40 mM-phosphate buffer, pH 7.5.

buffer was necessary for germination in methionine, but this was a consequence of pH changes induced by the decomposition of thioglycollate (Holland & Shoemith, 1971).

The effect of reducing agents

In most of this work thioglycollate was used to maintain anaerobic conditions. With some clostridia this compound is inhibitory (Treadwell, Jann & Salle, 1958; Gibbs, 1964) but not with *Clostridium tetani*. When germination took place in either heart-infusion broth, or methionine, lactate and nicotinamide, or leucine, phenylalanine, methionine, lactate and

Table 8. *The effect of thioglycollate on the germination of spores in a defined medium*

Thioglycollate (mM)	Time for 50 % (min)
0	41
0.1	29
1.0	26
10	24
100	24

Germination took place in the presence of methionine (1 mM), lactate (100 mM), nicotinamide (82 μ M), phosphate buffer (40 mM, pH 7.5) and Na⁺ (280 mM) at 37°.

nicotinamide similar results were obtained with either 10 mM-thioglycollate or 10 mM-ascorbate as the reducing agent. Cysteine was also effective. Similarly, spore suspensions bubbled with O₂-free N₂ (British Oxygen Company, Leeds, 12) germinated at the same rate whether thioglycollate was present or not. Different concentrations of thioglycollate up to 100 mM gave no inhibitory effect (Table 8).

DISCUSSION

Germination of *Clostridium tetani* spores is actively stimulated by a combination of 1 mM-methionine, 100 mM-lactate, 82 μ M-nicotinamide and 280 mM-Na⁺, all of which may be readily available in a natural environment such as dead or damaged tissue. Lactate and Na⁺ have already been noted as germinants for *Clostridium bifermentans* (Gibbs, 1964, 1967). These more complex germination requirements may be characteristic for *C. tetani* and therefore useful taxonomically in a similar manner to which Rode (1968) found that *Bacillus megaterium* could be divided into two types whose germination requirements correlated with other differences.

Other compounds, such as leucine and phenylalanine, have less activity for stimulating germination but are effective with other spores (Hitzmann *et al.* 1957; Gibbs, 1964). Among the other amino acids, alanine, known as a germinant for many other species, was inactive. Bicarbonate, though an active germinant for some clostridia (Holland *et al.* 1970), was slightly active with *Clostridium tetani* but only in the presence of leucine and phenylalanine. (The results with these less active germinants have been confirmed and extended in more detailed experiments—Holland, 1969; Holland & Shoesmith, 1971.)

Under aerobic conditions germination is slow and methionine, lactate, nicotinamide and Na⁺ are all necessary. With these compounds present under anaerobic conditions the time for 50 % germination is shortened by about 60 %. Anaerobic conditions also enable germination to take place either in lactate, nicotinamide and Na⁺ or in methionine and Na⁺, and, though this germination is slower than with all the compounds present, it is faster than the optimal aerobic germination. The germination system is therefore not insensitive to aerobic conditions, showing a response between that of spore viability, which is little affected by oxygen (J. G. Shoesmith, unpublished observations), and cell development, where anaerobic conditions are well known to be necessary for outgrowth and multiplication (Knight & Fildes, 1930).

A similar dependency of the specificity of germinants on environmental factors (aerobic or anaerobic conditions) has been found with strains of *Clostridium sporogenes* PA3679

(Holland *et al.* 1969) and Gibbs (1971) found that the presence of bicarbonate made heat-shock and phenylalanine no longer necessary for the rapid germination of *C. bifermentans*.

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