GERMINATION OF CLOSTRIDIUM SPORES IN BUFFERED GLUCOSE[1]

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It was reported by Schreiber (1896) that the elements carbon, hydrogen, oxygen, potassium, magnesium, phosphorus, and sulfur were necessary for germination of spores of 3 *Bacillus* species. According to Curran (1931) the minimal concentration of peptone permitting germination of *Bacillus mycoides* spores was between 0.02 and 0.025 per cent. On the other hand, evidence has been presented by Knaysi (1945) that some normal mature spores of *B. mycoides* are able to germinate when supplied solely with a utilizable source of energy such as glucose. To the authors' knowledge, no studies have appeared directly concerning minimal nutritional requirements for germination of spores of anaerobic species. Recently in this laboratory we have been concerned with the problem of devising a chemically defined medium for evaluation of possible effects on spore germination of carcinogens and substances used in chemotherapy of cancer. In the course of these investigations evidence has been obtained that spores of *Clostridium* species are capable of germination in buffered glucose solutions.

MATERIALS AND METHODS

Organisms used were *Clostridium perfringens*, *Clostridium chauvei*, putrefactive anaerobe, strain no. 3679, and 2 strains of *Clostridium botulinum* (62A and 115B), all from the Department of Bacteriology, University of Texas, Austin. The procedure of preparation of spore suspensions was given earlier (Wynne and Foster, 1948). For germination studies, two per cent glucose containing 0.02 M phosphate was used, with the phosphate autoclaved separately.

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Diluted suspensions containing about 1,000 to 10,000 viable spores per ml in distilled water were heated 5 minutes at 75 C for possible heat activation of the spores (Reynolds and Lichtenstein, 1949) and possible stabilization of the suspension by destruction of partially germinated or heat-labile spores (Stumbo *et al.*, 1950). One ml portions were inoculated into 9 ml of the buffered, glucose solution preheated to 75 C to insure proper oxidation-reduction potential for germination. Incubation for germination was at 37 C in an atmosphere of natural gas. After incubation, tubes were stored overnight in air atmosphere at 3 C to destroy any vegetative cells by contact with oxygen. Counts of residual spores ordinarily were made from duplicate platings from each of triplicate tubes so that each count represented an average of 6 replicates. The counting medium was used in Prickett tubes and consisted of modified Yesair's pork infusion agar containing 0.1 per cent starch, with an overlying anaerobic seal of 1.5 per cent agar-agar with BBL thioglycolate supplement (Wynne and Foster, 1948). Colony counts were made after 2 to 3 days of incubation at 37 C and rechecked up to 3 to 4 weeks with no significant increase. The over-all counting error was estimated at about ±10 per cent.

For morphological studies, the malachite green mercurochrome spore stain reported previously (Wynne, 1948) was used, with initial spore levels of around 500,000 per ml.

RESULTS AND DISCUSSION

Following incubation in buffered glucose there was a marked decrease in recoverable spores (table 1). The difference between the spore level following incubation in phosphate alone and that following incubation in phosphate plus glucose was assumed to represent germinated spores for the purpose of calculating per cent apparent germination, a term derived from the quotient of germinated spores and the

TABLE 1

Decrease in recoverable spores of Clostridium species on incubation at 37 C in buffered glucose

MEDIUM	INCUBATED	SPORE COUNTS IN MODIFIED YESAIR'S AGAR WITH STARCH				
		C. perfringens, 46 hr incubation	*C. chauvei*, 45 hr incubation	*C. botulinum*, strain 62A, 68 hr incubation	*C. botulinum*, strain 115B, 68 hr incubation	Putrefactive anaerobe, strain 3679, 48 hr incubation
Water	−	1,050	1,250	570	900	770
Phosphate, pH 7	+	1,070	1,250	560	720	—
Glucose + phosphate, pH 7	+	60 (94%)	125 (90%)	130 (77%)	150 (79%)	25 (97%)
Same + oleate, 1,000 μg per ml	+	970	1,090	550	760	—
Glucose + phosphate, pH 4.8	+	810	—	530	620	—

(—%) = per cent apparent germination, e.g., with *Clostridium perfringens* $\left(\frac{1070-60}{1070}\right)100 = 94\%$

TABLE 2

Lack of effect of postincubation treatment on apparent germination in buffered glucose

TREATMENT	INCUBATED	SPORE COUNTS IN MODIFIED YESAIR'S AGAR WITH STARCH				
		C. botulinum, strain 115B	*C. botulinum*, strain 62A	*C. perfringens*	*C. chauvei*	Putrefactive anaerobe, strain 3679
None	−	840	410	560	710	140
None	+	24 (97%)	40 (90%)	0 (100%)	2 (99+%)	22 (84%)
3 C overnight	+	10 (99%)	32 (92%)	0 (100%)	2 (99+%)	2 (99%)
75 C for 20 min in BHI	+	24 (97%)	34 (92%)	1 (99+%)	3 (99+%)	6 (96%)
Same	−	820	420	560	750	150

BHI = Brain heart infusion broth.

(—%) = per cent apparent germination calculated as in table 1.

spore count following incubation in phosphate buffer. That the marked decrease in spore counts following incubation in neutral buffered glucose was actually due to germination was indicated by the following: (1) No significant change in counts occurred in the 4 strains tested in the presence of 1,000 μg per ml of added oleate, a known inhibitor of germination in these organisms (Wynne and Foster, 1948; Roth and Halvorson, 1952). (2) Counts were practically unaffected in the 3 strains studied at pH 4.8. Although this pH was not tested with *C. chauvei*, spore counts following incubation at pH 6.7 were considerably higher than those obtained at pH 7. (3) Some decrease in counts was observed following incubation in air with *C. perfringens*, *C. chauvei*, and putrefactive anaerobic, strain no. 3679, but not with *C. botulinum*, strain 115B. Slow germination in complex media under aerobic conditions has been described for the first 3 species (Wynne and Harrell, 1951; Wynne *et al.*, 1952). (4) The decrease in countable spores of putrefactive anaerobe, strain no. 3679, was apparently logarithmic in keeping with the previously reported kinetics of germination for this organism in a complex medium (Mehl and Wynne, 1951). (5) Stained smears prepared from tubes containing initial spore levels around 500,000 per ml showed that incubation in buffered glucose resulted in significant decreases in spore percentages in *C. chauvei* and *C. perfringens*.

It will be noted that the usual criterion of germination employed in previous studies (Wynne and Foster, 1948), the change from a thermoresistant spore to a thermolabile entity, was not used in these studies. Instead, tubes were stored overnight at 3 C to destroy any vegetative cells present. However, when no postincubation treatment was used, residual spore counts, and hence germination by difference, were similar to those obtained following

refrigeration or heating in brain heart infusion broth (table 2). Apparently germinated spores either perished from nutritional inadequacy of the medium or during the manipulations of dilution and counting.

SUMMARY

Physiological and morphological evidence has been presented that germination of spores of 5 clostridium strains occurs in glucose buffered with phosphate. In agreement with previously reported studies with complex media, this germination was sensitive to effects of oleate, acid pH, and unfavorable Eh; and germination in putrefactive anaerobe, strain 3679, was apparently logarithmic.

REFERENCES

CURRAN, H. R. 1931 Influence of osmotic pressure upon spore germination. J. Bact., **21**, 197–209.

KNAYSI, G. 1945 Investigation of the existence and nature of reserve material in the endospore of a strain of *Bacillus mycoides* by an indirect method. J. Bact., **49**, 617–622.

MEHL, D. A., AND WYNNE, E. S. 1951 A determination of the temperature characteristic of spore germination in a putrefactive anaerobe. J. Bact., **61**, 121–126.

REYNOLDS, H., AND LICHTENSTEIN, H. 1949 Germination of anaerobic spores induced by sublethal heating. Soc. Am. Bact. Abst. of Papers, 49th Gen. Meeting, p. 9.

ROTH, N. G., AND HALVORSON, H. O. 1952 The effect of oxidative rancidity in unsaturated fatty acids on the germination of bacterial spores. J. Bact., **63**, 429–435.

SCHREIBER, O. 1896 Ueber die physiologischen Bedingungen der endogenen Sporenbildung bei *Bacillus anthracis*, *subtilis*, und *tumescens*. Zentr. Bakteriol. Parasitenk., Abt. I, Orig., **20**, 353–374, 429–437.

STUMBO, C. R., MURPHY, J. R., AND COCHRAN, T. 1950 Nature of thermal death time curves for P.A. 3679 and *Clostridium botulinum*. Food Technol., **4**, 321–326.

WYNNE, E. S. 1948 Physiological studies on spore formation in *Clostridium botulinum*. J. Infectious Diseases, **83**, 243–249.

WYNNE, E. S., AND FOSTER, J. W. 1948 Physiological studies on spore germination with special reference to *Clostridium botulinum*. I. Development of a quantitative method. J. Bact., **55**, 61–68.

WYNNE, E. S., AND HARRELL, K. 1951 Germination of spores of certain *Clostridium* species in the presence of penicillin. Antibiotics & Chemotherapy, **1**, 198–202.

WYNNE, E. S., COLLIER, R. E., AND MEHL, D. A. 1952 Locus of action of streptomycin in the development of clostridia from spore inocula. J. Bact., **64**, 883–886.