SPORULATION OF *CLOSTRIDIUM BOTULINUM* TYPES A, B, AND E, *CLOSTRIDIUM PERFRINGENS*, AND PUTREFACTIVE ANAEROBE 3679 IN DIALYSIS SACS[1]

MORRIS D. SCHNEIDER,[2] NICHOLAS GRECZ, AND ABE ANELLIS

Quartermaster Food and Container Institute for the Armed Forces, Quartermaster Research and Engineering Command, U.S. Army, Chicago, Illinois

Received for publication 4 August 1962

ABSTRACT

SCHNEIDER, MORRIS D. (Quartermaster Food and Container Institute for the Armed Forces, U.S. Army, Chicago, Ill.), NICHOLAS GRECZ, AND ABE ANELLIS. Sporulation of *Clostridium botulinum* types A, B, and E, *Clostridium perfringens*, and Putrefactive Anaerobe 3679 in dialysis sacs. J. Bacteriol. **85**:126–133. 1963.—Concentrated cultures of spores of *Clostridium botulinum* type A (33A, 37A), B (41B, 51B), and E (strain VH), *C. perfringens* (strain E), and Putrefactive Anaerobe 3679 were prepared in intussuscepted cellulose dialysis tubing. The apparatus consisted of a telescoped cellulose bag immersed into a suitable sporulation medium in a large Pyrex tube. The initial inoculum was a heavy suspension in physiological saline solution of either vegetative cells or heat-shocked spores. The seed material was introduced into the interior of the dialysis bag. Maximal spore populations were obtained within 10 to 12 days. Strains of *C. botulinum* type E and *C. perfringens*, known for their poor sporulation in conventional cultures, gave good spore crops in the dialysis bag. Some crops were of the order of 10^{10} and 10^{11} viable spores per liter of medium. The spores produced in the dialysis bag were conspicuously large, particularly after incubation for 20 to 30 days. Observations of the characteristics of spores formed in telescoped bags indicate that two highly resistant strains of *C. botulinum*, 33A and 41B, were apparently less resistant to gamma rays than spores of the same strains produced in identical media in conventional cultures.

[1] Approved for publication as paper no. 2222 by the Armed Forces Food and Container Institute, Quartermaster Research and Engineering Command, U.S. Army.

[2] Present address: Armour Research Foundation of the Illinois Institute of Technology, Chicago.

Propagation of bacteria in cellophane tubes immersed in an appropriate nutrient medium results in vigorous growth and concentration of macromolecular products and cells in a confined environment (Gallup and Gerhardt, 1961). Toxigenic strains of *Clostridium botulinum* and *C. tetani* have been grown in dialysis sacs because of the high titers of toxin produced in a restricted volume in this system (Sterne and Wentzel, 1950; Wentzel, Sterne, and Polson, 1950; Vinet and Fredette, 1951; Fredette and Vinet, 1952; Gerwing, Dolman, and Arnott, 1961). Media enriched with corn-steep liquor especially stimulate abundant vegetative cell growth, including the synthesis of high titers of botulinum toxins (Lamanna, Eklund, and McElroy, 1946; Polson and Sterne, 1946).

The formation of bacterial spores in dialysis-sac cultures has not previously been reported. In this laboratory, it was observed that under certain conditions large yields of spores may be obtained from various species of clostridia, including some which are known for their poor sporulation properties. This paper describes the apparatus, media, spore yields, and some properties of spores produced in dialysis-sac cultures.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains included *C. botulinum* 33A, 37A, 41B, and 51B, the sources and radiation-resistance characteristics of which were reported by Anellis and Koch (1962); *C. botulinum* type E strain Dolman VH; Putrefactive Anaerobe (PA) 3679; and *C. perfringens*, strain E_3.

Media. The following media were tested for their ability to support sporulation: (i) the corn "steepwater" fluid medium of Sterne and Wentzel (1950); (ii) the medium of Stewart (*personal communication*) that contains 6.5% Trypticase (BBL), 1.0% peptone, 0.25% NaCl, 0.25%

K_2HPO_4, 0.2% methionine, and 0.02% thiamine (pH 8), in which the stock medium, and the methionine and thiamine were sterilized separately and combined after sterilization; (iii) Wagenaar and Dack's (*personal communication*) broth containing 5.0% Trypticase and 0.5% peptone (pH 7.2); (iv) the broth of Costilow (*personal communication*) consisting of 4% Trypticase and 0.01% thiamine (pH 7.0); and (v) a broth (pH 6.85) of Wagenaar and Dack modified by enrichment with thioglycollate supplement (BBL).

The apparatus consisted essentially of a telescoped cellulose bag immersed into the sporulation medium in a large Pyrex tube (Fig. 1).

Description of the culture apparatus. Glass culture tubes (Pyrex) with lips and medium-weight walls were used in two sizes: small (400-mm length by 50-mm outside diameter) and large (500-mm length by 65-mm outside diameter). The tubes were fitted with a no. 10 (small) or no. 13 (large) three-holed rubber stopper. The smallest of the three holes was fitted with a straight Pyrex tube (6 mm in diameter and 450 or 550 mm long) plugged with cotton, the lower open end of which was immersed in the medium to about 25 mm from the bottom of the flask. A second hold in the rubber stopper was fitted with a straight tube (10 by 150 mm) which was cotton-plugged at its top end. The open lower end extended to 100 to 150 mm above the surface of the medium inside the culture flask. The 10-mm tube allowed release of gas pressure built up during sparging of the medium and cellular growth. A third hole was fitted with a Pyrex tube (15 by 250 mm); its cotton-plugged upper end (about 50 mm) was bent at an angle of 45°. The lower end of this tube extended to 25 to 50 mm above the level of the medium in the flask. This lower or open end of the 15-mm tube was inserted into the ring space inside an intussuscepted cellulose dialysis tube.

The telescoped tube was formed as follows. A 750-mm length of tubing (E. H. Sargent & Co., Chicago, Ill.) was softened in water, and one end tied securely with a figure-eight knot. The addition of a small amount of water facilitated telescoping, the knotted end being inverted and pushed through to the untied end of the cellulose tube. The telescoped bag was tested for leaks. It was then securely fixed to the 15-mm Pyrex tube by three rubber bands. The telescoped bag thus

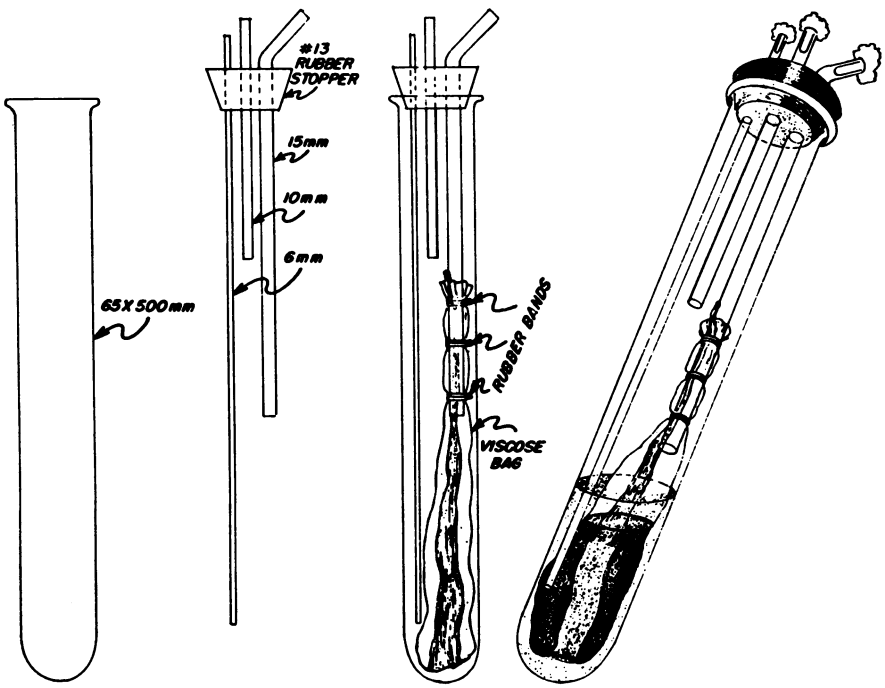

FIG. 1. *Apparatus for concentrated culture and sporulation of Clostridium spp.*

formed was about 375 mm in length and 65 mm in diameter. It was straightened to eliminate folds and to give the maximal dialyzing surface.

Final assembly and sterilization of apparatus. The inner space of the intussuscepted bag was wetted with 25 ml of physiological saline solution, and the open end of the 15-mm bent tube fitted with a cotton plug. The three-hole rubber stopper and the inverted dialysis-tube assembly were inserted into the Pyrex culture flask, followed by addition of 200 or 350 ml of appropriate medium for the small and large flasks, respectively. Two paper strips were placed between the lip of the Pyrex tube and the rubber stopper, to preclude cementing of the rubber stopper to the glass. Kraft paper covered the top of the assembly to prevent accidental contamination of the interior contents. The culture flask was placed in a large-sized wire basket at a 45° angle. This minimized boiling off of the medium during sterilization, as well as allowing escape of air bubbles trapped inside the telescoped bag. The apparatus was sterilized at 121 C for 30 min. After autoclaving, the paper inserts between the glass tube and rubber stopper were removed while the apparatus was still hot.

Figure 2 shows three Pyrex culture flasks containing a 7-day growth of *C. botulinum* in Wagenaar and Dack's broth. Microbial growth is restricted entirely to the saline fraction inside the viscose bag.

Seed materials. (i) Vegetative cells: 30-ml cultures were transferred serially at 2-hr intervals to obtain vigorous vegetative growth, and inoculated into 350 ml of the appropriate medium. Young cells (5 to 6 hr old) were centrifuged and resuspended in 50 ml of sterile saline solution. (ii) Spore seed: spores were derived from crops produced in viscose-bag cultures. The spores were either the entire unwashed saline-dialyzed culture or thrice-washed spores resuspended in a final volume of 50 ml of saline. Spore suspensions were in all instances (excepting *C. botulinum* type E) heat-shocked at 80 C for 10 min to kill residual vegetative cells and to activate the spores to germination. The seed culture was transferred aseptically to the intussuscepted cellophane sac by means of a sterile 50-ml pipette.

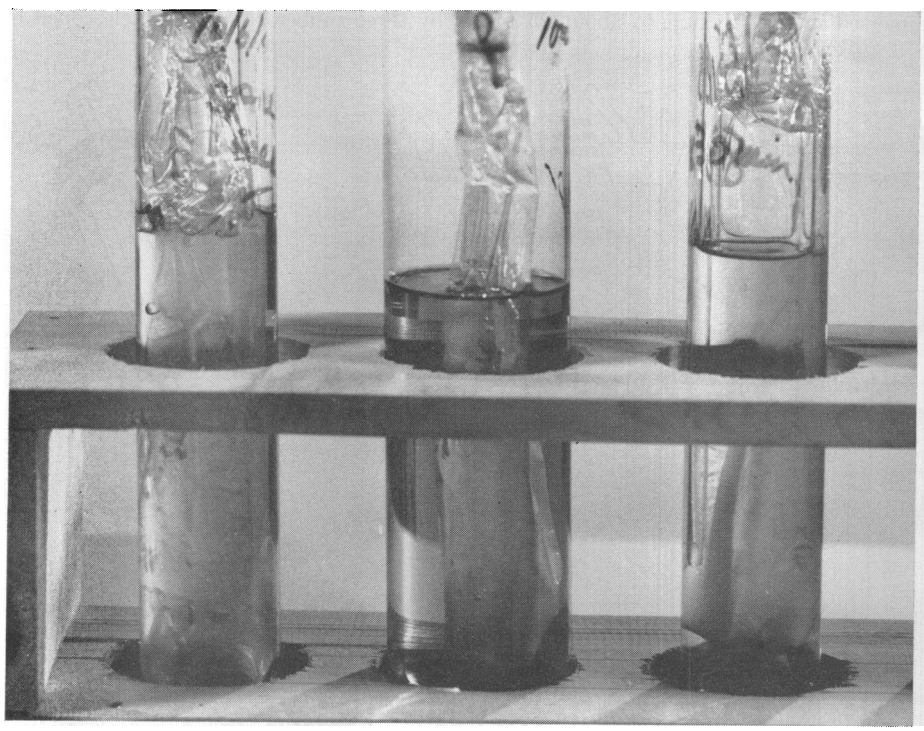

FIG. 2. *Culture tubes containing a 7-day growth of Clostridium botulinum in Wagenaar and Dack's broth.*

Anaerobiosis. A mixture of 95% N_2 and 5% CO_2 was passed through a cotton filter. The gas mixture was bubbled through the medium by way of the long tubing (6-mm diameter) for 5 min before and 10 min after introduction of the seed material into the viscose bag, to create anaerobic conditions in the medium.

Control cultures. Conventional cultures of *C. botulinum* 33A, 41B, and 51B were grown in 350 ml of appropriate medium in 500-ml Erlenmeyer flasks, placed in desiccators. Oxygen was removed by a pyrogallol-sodium carbonate solution. In experiments involving *C. botulinum* 37A, Erlenmeyer flasks (4 liters) containing 3 liters of Costilow's broth were seeded with an entire 350-ml culture of cells 5 to 6 hr old. Anaerobiosis was achieved by sparging the 3 liters of medium with the 95% N_2-5% CO_2 mixture for 15 min each before and after inoculation.

Harvesting saline-dialyzed spore crops. The fitted rubber stopper, together with the dialysis sac, was lifted out of the medium. The culture assembly was held over a sterile petri dish, while nearly three-fourths of the width of the telescoped tube was clamped securely with a sterile surgical hemostat just below the 15-mm glass tube. The lower end of the dialysis bag was grasped by hand with sterile aluminum foil. The sedimented spores were thoroughly mixed, the bag was cut with sterile scissors above the clamped hemostat, and the contents were poured into a sterile centrifuge bottle. The empty bag was washed with 20 ml of sterile phosphate buffer to recover the total spore crop. Two 240-ml centrifuge bottles were sufficient to harvest the spores from ten dialysis-bag assemblies. The suspension was centrifuged at 5 to 8 C at approximately 1,500 $\times$ *g* for at least 60 min, and washed three times with sterile phosphate buffer (pH 7.0). The spores were resuspended in 100 ml of buffer and held at 5 C.

Estimation of viable spore numbers. The stock spore suspensions were heat-shocked at 80 C for 10 min, except for the heat-sensitive type E *C. botulinum* strain VH spores, which were heated at 65 C for 15 min. Serial decimal dilutions were made in neutral phosphate buffer; 1-ml samples of each dilution were inoculated in each of five replicate tubes of Wynne's (Wynne, Schmeiding, and Daye, 1955) broth. Growth in the medium was usually completed by 14 days, but cultures were kept under observation at 30 C for 6 weeks. The most probable number of viable spores was calculated from the statistical tables of Fisher and Yates (1953).

Toxin assay. Botulinum toxin in dialyzed supernatant fluids was assayed by duplicate intraperitoneal injections into white mice (16 to 20 g). A positive reaction was indicated by death of the mice within 4 days. The specific types of toxin were confirmed by toxin-antitoxin neutralization tests. Tenfold serial dilutions were made of the dialyzed supernatant fluids in a gelatin-phosphate diluent (Naylor and Smith, 1946). Gelatin protein appears to minimize inactivation of the toxin, reduce pyrogenic reactions, and potentiate the effect of botulinum toxin (Wentzel et al., 1950; Boor, Tresselt, and Shantz, 1955).

Radiation resistance of Clostridium botulinum spores produced in dialysis sacs. Spore crops produced in conventional cultures were compared with those produced in telescoped viscose tubes. The broth of Wagenaar and Dack was used as the sporulating medium in both instances. Approximately 10^4 viable spores, suspended in 1-ml volumes of phosphate buffer and using ten replicate tubes per dose, were irradiated with spent fuel rods at the Argonne National Laboratory Gamma Irradiation Facility. The methods of irradiation and evaluation of results were those described by Anellis and Koch (1962).

RESULTS

Three formulations of Trypticase-containing media promoted the formation of remarkable numbers of spores of *C. botulinum* 37A. Furthermore, saline-dialysis sac cultures contained sizeable amounts of preformed botulinum toxin. A corn "steepwater" medium of Sterne and Wentzel (1950) resulted in the synthesis of a high titer of botulinum toxin and, at the end of 21 days of incubation, revealed large numbers of vegetative cells and smaller numbers of spores (Table 1). The broth of Wagenaar and Dack appeared to yield the highest spore crop of the media tested, producing approximately 10^8 spores per ml of the dialysis-sac culture in the bag within 10 days of incubation (Table 2). Bio-assay of a number of preparations of the crude, "spore-free" botulinum toxin in the growth medium gave titers of 10,000

to 100,000 mouse lethal doses per ml for strains 41B, 51B, and 33A.

Comparative sporulating ability of C. botulinum 37A in conventional cultures and in telescoped viscose tubes in a single medium. The data obtained for *C. botulinum* 37A grown in the medium of Costilow are shown in Table 3. The number of spores harvested after 5 to 24 days from the dialysis bag was nearly 100-fold higher than the number of spores produced by the conventional method in this medium. This broth gave abundant growth and produced 100,000 minimal lethal doses (MLD)/ml of botulinum toxin in 3 days of incubation. The toxin degraded to 1,000 MLD at the end of 24 days. This was apparently due to the relatively high pH in the medium (pH 8.0 to 8.15), since it is known that botulinum toxin is not stable in alkaline solutions (Bronfenbrenner and Schlesinger, 1924). However, the increase in alkalinity in the "spent" medium did not interfere with the sporulation process.

Sporulation of C. botulinum type E, C. perfringens, and Putrefactive Anaerobe 3679 in dialysis sacs. Several spore-forming anaerobes other than *C. botulinum* types A and B produced good spore crops in dialysis sacs immersed into the medium of Wagenaar and Dack fortified with 0.5% thioglycollate supplement (Table 4). Noteworthy are the particularly abundant spore crops for PA 3679, as well as excellent spore crops for type E, *C. botulinum* strain VH, and *C. perfringens* strain E_3. The latter two strains are generally known for their poor sporulation in conventional cultures. For example, optimal conditions for growth of *C. perfringens* require the presence of a fermentable carbohydrate, but spores form sparingly and only in the absence of a fermentable carbohydrate (Jordan and Burrows, 1947). It is of significance that *C. perfringens* grew luxuriantly in the dialysis-sac sys-

TABLE 1. *Sporulation of Clostridium botulinum 37A in various media at 30 C*

Medium	Days of incubation	Nondialyzable saline culture	
		MPN of viable spores per liter of medium	Potency*
			MLD/ml
Sterne and Wentzel (1950)	21	7.4×10^8	1,000,000
Stewart	26	1.2×10^{10}	10,000
Waganaar and Dack	33	6.8×10^{10}	10,000
Costilow	3	3.52×10^8	10,000
	20	1.73×10^9	1,000
	69	5.69×10^9	1,000

* Of the "spore-free" culture-centrifugate from within the dialysis sack.

TABLE 2. *Sporulating ability of three strains of Clostridium botulinum in cellulose dialyzing tubes immersed in the broth of Wagenaar and Dack**

Strain	Days of incubation	MPN of viable spores in culture medium	
		Per ml of spore harvest	Per liter of medium
33A	10	7.37×10^8†	1.76×10^{10}
33A	10	4.50×10^8†	1.90×10^{10}
41B	3	2.71×10^7	4.34×10^9
41B	10	1.73×10^8	4.07×10^9
41B	10	1.73×10^8†	1.73×10^{10}
41B	4	2.71×10^6	3.86×10^8
41B	10	1.73×10^8†	6.19×10^9
41B	19	7.37×10^7†	5.26×10^9
51B	3	7.37×10^5	1.94×10^8
51B	10	2.81×10^8†	6.0×10^9
51B	7	7.37×10^7	1.10×10^{10}
51B	10	1.14×10^9	3.93×10^{10}

* Broth contains 5% Trypticase + 0.5% peptone.

† Crops of spores washed three times and resuspended in neutral phosphate buffer to 100-ml volume.

TABLE 3. *Comparative sporulating ability of Clostridium botulinum 37A in conventional culture* (C) *and in cellulose dialysis tubes* (S) *in Costilow's broth at 30 C**

Culture	Days of incubation	MPN of viable spores in culture medium		Potency†
		Per ml of spore harvest	Per liter of medium	
				MLD/ml
C	3	4.36×10^5	4.36×10^8	1,000
S	3	2.71×10^6	7.10×10^8	100,000
C	5	1.73×10^5	1.73×10^8	1,000
S	5	4.50×10^7	9.0×10^9	10,000
C	12	4.50×10^6	4.50×10^9	1,000
S	12	2.71×10^8	1.83×10^{10}	10,000
C	24	2.71×10^6	2.71×10^9	100
S	24	1.14×10^8	2.28×10^{10}	1,000

* Contains 4% Trypticase, 0.01% thiamine.

† Of "spore-free" toxic culture.

TABLE 4. *Sporulation in dialysis sacs of Clostridium botulinum 33A, C. botulinum type E, C. perfringens, and Putrefactive Anaerobe 3679 at 30 C*

Culture	Days of incubation	MPN of viable spores		Range of "spent" medium (pH)
		Per ml of spore harvest	Per liter of medium*	
C. botulinum 33A	11	1.73×10^8	2.71×10^{10}	6.3 (3)‡
C. botulinum 33A	12	1.73×10^8†	6.17×10^9	6.45–6.71 (10)
C. botulinum E				
Expt 1				
Heated (65 C for 15 min)	11	1.73×10^6	2.60×10^8	6.5 (2)
Raw	11	1.14×10^6		
Heated (80 C for 10 min)	11	1.14×10^3		
Expt 2				
Heated (65 C for 15 min)	15	2.71×10^7†	7.73×10^8	6.30–6.48 (10)
Heated (80 C for 10 min)	15	4.50×10^4		
C. perfringens	6	7.37×10^6	9.11×10^8	6.50–6.78 (3)
C. perfringens	15	7.37×10^7†	2.34×10^9	6.42–6.60 (9)
PA 3679	15	2.71×10^9	3.87×10^{11}	6.50–6.70 (2)
PA 3679	13	1.73×10^9†	6.17×10^{10}	6.7–6.9 (9)

* Modified Wagenaar and Dack's medium: 5% Trypticase, 0.5% peptone, and 0.5% thioglycollate supplement (BBL).

† Crops of spores washed three times and resuspended in neutral phosphate buffer to 100-ml volume.

‡ Figure in parentheses is number of culture flasks.

tem and formed abundant spore crops in the presence of a fermentable carbohydrate. The saccharolytic tendency of the strains of *C. botulinum* type E and *C. perfringens* was indicated by a slight decrease in pH of the "spent" medium.

Spores of *C. botulinum* strain VH produced in telescoped sacs did not appear to become more resistant to heating than those produced in conventional culture.

Morphology of C. botulinum and spores in dialysis sacs. Cultures of *C. botulinum* in dialysis sacs showed unusual microscopic morphology not observed in conventional cultures, such as chains of sporangia containing well-formed spores; "dumb-bell" type sporangia; and conspicuously large spores, particularly after a long incubation time (20 days or longer).

Certain strains of *C. botulinum*, such as 33A and 51B, appear to possess distinguishing morphological features of uniformly large (33A) and small (51B) spore sizes.

The small spores of 51B in conventional cultures were difficult to observe by simple stains. Spores of this strain produced in dialysis sacs were relatively large and easily recognizable by crystal violet stain.

The spores seemed to be surrounded by wide semiclear zones at their surfaces, after staining with nigrosin. The spores also aggregated into clumps after heating at 80 C for 10 min, suggesting, perhaps, that nondialyzable substances in the bag were coagulated or that some sticky material may have been adhering to the spore surfaces.

TABLE 5. *Radiation resistance of Clostridium botulinum spores formed in viscose dialysis sacs**

Spore strain	Expt no.	Radiation resistance		
		D value	Avg D	Deviation
		Mrad	*Mrad*	%
In dialysis sacs				
33A	1	0.283	0.248	14.0
33A	2	0.212		
41B	1	0.266		
41B	2	0.256	0.261	1.8
In conventional cultures				
33A	1	0.338	0.334	1.1
33A	2	0.330		
41B	1	0.301		
41B	2	0.334	0.318	5.2

* Approximately 10^4 viable spores, previously heat-shocked at 80 C for 10 min, in 1-ml volume of phosphate buffer (pH 7).

Radiation resistance. In view of the morphological differences exhibited by spores prepared by the two methods, it was of interest to compare

their relative radiation resistances. The procedure used for this study was that described by Anellis and Koch (1962).

D values, i.e., the doses required to kill 90% of the population, were determined in duplicate experiments for 10-day-old spores of *C. botulinum* 33A and 41B, two of the most radiation-resistant organisms of their respective types (Anellis and Koch, 1962). The data (Table 5) indicate that both strains of spores produced in the dialysis sac exhibited a somewhat reduced resistance to radiation as compared with conventionally produced spores; the resistance of 33A decreased by 26%, and that of 41B by 18%. The reasons for the reduced resistance of the dialysis-bag harvests are not yet clear.

DISCUSSION

Abundant spores of clostridia can be produced in cellophane casing immersed in an appropriate nutrient medium. The technique reduces the hazard involved in handling large volumes of toxigenic *C. botulinum* cultures, and eliminates laborious centrifugations required for harvesting of spores by the conventional method. Furthermore, the technique is suitable for the production of spores of some clostridia known for their poor sporulation in conventional cultures, such as *C. botulinum* type E and *C. perfringens*.

The events leading to the formation of bacterial spores in the dialysis-sac system are not yet understood. The properties of the dialysis membrane suggest at least four factors which may be involved in sporogenesis in this environment: (i) selective supply of low molecular size (mol wt <13,000) nutrient compounds through the ultrafine pores (0.0024 to 0.003 μ) of the dialysis membrane; (ii) accumulation of macromolecular metabolic products which may contain some agent(s) essential for the triggering of abundant sporogenesis; (iii) removal of low molecular weight metabolic waste products from the confined growth environment, which might normally interfere with sporogenesis; and (iv) the equilibrium rates involved in diffusion of materials across the dialysis membrane and, perhaps, affecting the chemical equilibria of metabolic processes within the bag.

In any event, the dialysis-bag technique seems to offer a challenging system for the study of physiological and chemical changes during sporulation occurring in the complete absence of any contact with the macromolecuar constituents of complex nutrient media.

ACKNOWLEDGMENTS

We wish to thank Albert A. Walker and Harry T. Lipsky for their technical assistance. Thanks are also due to Ruth L. Henoch for assistance in preparation of the manuscript.

LITERATURE CITED

ANELLIS, A., AND R. B. KOCH. 1962. Comparative resistance of strains of *Clostridium botulinum* to gamma rays. Appl. Microbiol. **10**:326–330.

BOOR, A. K., H. B. TRESSELT, AND E. J. SHANTZ. 1955. Effects of salts and colloids on the potency of botulinum toxin. Proc. Soc. Exptl. Biol. Med. **89**:270–272.

BRONFENBRENNER, J. J., AND M. J. SCHLESINGER. 1924. The effect of digestive juices on the potency of botulinus toxin. J. Exptl. Med. **39**:509–516.

FISHER, R. A., AND F. YATES. 1953. Statistical tables for biological, agricultural and medical research, 4th ed. Hafner Publishing Co., New York.

FREDETTE, V., AND G. VINET. 1952. Production of tetanus toxins containing at least 500,000 lethal (guinea pig) doses per millilitre in cellophane bags. Can. J. Med. Sci. **30**:155–156.

GALLUP, D. M., AND P. GERHARDT. 1961. Concentrated culture of bacteria in dialysis flask and fermentor systems. Bacteriol. Proc., p. 52.

GERWING, J., C. E. DOLMAN, AND D. A. ARNOTT. 1961. Purification and activation of *Clostridium botulinum* type E toxin. J. Bacteriol. **81**:819–822.

JORDAN, E. O., AND W. BURROWS. 1947. Textbook of bacteriology, p. 525, 14th ed. W. B. Saunders Co., Philadelphia.

LAMANNA, C., H. W. EKLUND, AND O. E. MCELROY. 1946. Botulinum toxin (type A); including a study of shaking with chloroform as a step in the isolation procedure. J. Bacteriol. **52**:1–13.

NAYLOR, H. B., AND P. A. SMITH. 1946. Factors affecting the viability of Serratia marcescens during dehydration and storage. J. Bacteriol. **52**:565–573.

POLSON, A., AND M. STERNE. 1946. Production of potent botulinum toxins and formol-toxoids. Nature **158**:238–239.

STERNE, M., AND L. M. WENTZEL. 1950. A new method for the large-scale production of high-

titre botulinum formol-toxoid types C and D. J. Immunol. **65**:175–183.

VINET, G., AND V. FREDETTE. 1951. Apparatus for the culture of bacteria in cellophane tubes. Science **114**:549–550.

WENTZEL, L. M., M. STERNE, AND A. POLSON. 1950. High toxicity of pure botulinum type D toxin. Nature **166**:739–740.

WYNNE, E. S., W. R. SCHMIEDING, AND G. T. DAYE, JR. 1955. A simplified medium for counting *Clostridium* spores. Food Res. **20**:9–12.