

Isolation of *Clostridium botulinum* Type G from Swiss Soil Specimens by Using Sequential Steps in an Identification Scheme

WOLFGANG F. SONNABEND,*† UDO P. SONNABEND, AND THOMAS KRECH‡

Institute of Medical Microbiology, St. Gallen, Switzerland

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After *Clostridium botulinum* type G organisms and toxin were identified in necropsy specimens in cases of unexplained death in adults and infants (O. Sonnabend, W. Sonnabend, R. Heinzle, T. Sigrist, R. Dirnhöfer, and U. Krech, *J. Infect. Dis.* 143:22-27, 1981), extensive research to detect *C. botulinum* type G in soil samples from Switzerland was done. A total of 41 specimens from virgin soil and from cultivated land were examined for the presence of *C. botulinum* type G and other toxin types. Because of the lack of the lipase marker in type G, the detection of *C. botulinum* type G was based on the demonstration of type G organisms in enrichment cultures by a type G-specific enzyme-linked immunosorbent assay to detect both the type G toxin and antigen; enrichment cultures in which type G toxin or antigen was identified by enzyme-linked immunosorbent assay were then tested by a type G-specific gel immunodiffusion agar procedure. This method not only isolated strains of type G but also strains of *Clostridium subterminale*, a nontoxic variant of *C. botulinum* type G. As a consequence of the observed cross-reactions caused by strains of *C. subterminale* within this test system, all isolates of type G had to be definitively confirmed by mouse bioassay. The sequential steps of these methods seem to be very useful for detecting *C. botulinum* type G organisms. *C. botulinum* type G strains were isolated in five soil samples from different locations in close association with cultivated land. The strains were found together with a type B strain, with types B and C, with types B and F, with types A and B, and with a nonproteolytic strain of type B, in one sample each. *C. botulinum* of types A, B, C, F, and G, with type B being the most common, was found in 18 (43.9%) of the 41 soil samples.

Clostridium botulinum type G has been isolated until now only twice from soil samples from Argentina, for the first time in 1969 (7). Recently, we identified *C. botulinum* type G and its associated neurotoxin in necropsy specimens in cases of unexplained death in adults and infants in Switzerland (20, 21). The isolates from the two countries are clearly different serologically (8) but harbor a plasmid of identical molecular weight (23). In one of the sudden infant death cases, we isolated *C. botulinum* type G from house dust, as well as from soil samples from houseplants and the garden (21). The epidemiological background of these findings was not clear, since there is little information about the incidence and the distribution of *C. botulinum* in soil specimens from Switzerland. Sixteen samples of soil and dirt from the cantons Tessin and Bern were sent to California in 1922; Meyer and Dubovsky demonstrated type B strains in five of the samples (13). No other thorough survey has ever been done. Therefore, we examined soil specimens from different areas of Switzerland for the incidence and distribution of *C. botulinum*, with special regard to type G.

C. botulinum type G is difficult to recognize in mixed cultures because of its lack of the lipase marker and its low toxigenicity. Unlike all other types of *C. botulinum*, type G does not produce the enzyme lipase. Also, it produces only small amounts of toxin in pure culture. Its toxin is potentiated by trypsin, as is the toxin from the nonproteolytic strains of types B and F and all strains of type E. Usually, a culture procedure employing a spore selection technique is used for isolating *C. botulinum*. One of the major disadvan-

tages of this procedure is that strains of *C. botulinum* which do not readily sporulate (e.g., *C. botulinum* type G) may not be isolated (19). That its presence has never been established in any food poisoning outbreak or in any food may be due to a lack of suitable detection methods. The use of a fluorescent-antibody technique and an enzyme-linked immunosorbent assay (ELISA) to detect and identify this toxin type in fecal specimens and enrichment cultures has been described previously (8, 11).

In our study, detection of *C. botulinum* type G was based on identification of type G organisms in enrichment cultures by the ELISA method to detect both toxic and nontoxic strains of type G (11). Incubated enrichment cultures in which type G toxin or antigen was identified by ELISA were tested by a gel immunodiffusion agar procedure (6) for isolating strains of *C. botulinum* type G. In this test system, the occurrence of cross-reactions with strains of *C. subterminale*, a nontoxic variant of *C. botulinum* type G (4), has been described previously (11), but serological tests (neutralization tests) are still necessary to ascertain the type of *C. botulinum* in mice. A comparative study was conducted to determine the conditions for toxin production of these identified strains of *C. botulinum* type G compared with those isolated from autopsies of cases of unexplained death in Switzerland (20, 21).

MATERIALS AND METHODS

Collection of specimens. Collection stations were selected to give representative samplings of a given geographical area. Specimens were taken to obtain information on the north-south distribution in relation to soil properties. Of the 41 specimens taken, 20 were from virgin soil on mountain slopes, ca. 1,000 m above sea level, from the central and the southern regions. The 21 soil samples collected in the northern part were mainly from cultivated land; of these, 7

* Corresponding author.

† Present address: OMNILAB, D-4000 Düsseldorf 13, Federal Republic of Germany.

‡ Present address: Institute of Medical Microbiology, University of Düsseldorf, D-4000 Düsseldorf, Federal Republic of Germany.

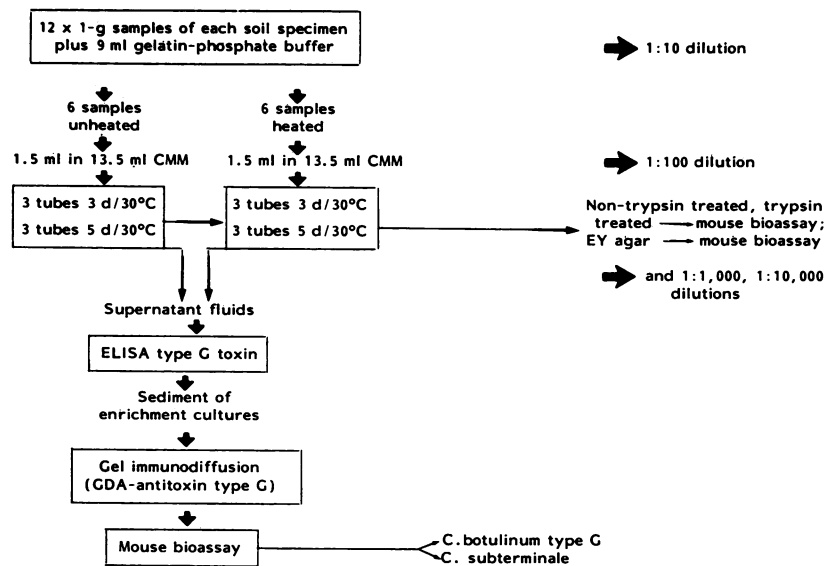


FIG. 1. Procedure for demonstration of *C. botulinum* type G organisms and toxin in soil samples. Abbreviations: CMM, cooked-meat medium; EY, egg yolk; GDA, gel diffusion agar; d, days.

were taken near stream banks and lake shores. One 150- to 200-g sample was obtained at each station with a sterile stainless spoon by removing and discarding the top 5 cm of soil and vegetation remains and sampling at a depth of 15 cm. Each specimen was placed in a plastic bag, transported to the laboratory without further manipulation, and stored at 4°C.

Demonstration of *C. botulinum*. The method used, which remained constant throughout, was previously described by Smith (16) with some modifications (Fig. 1). Twelve 1-g samples from each soil specimen were diluted 1:10 to 1:10,000 in gelatin-phosphate buffer and inoculated, with and without spore selection (heating at 70°C for 10 min), into tubes of cooked-meat medium which were incubated anaerobically at 30°C for 3 to 5 days. Incubated enrichment cultures were centrifuged at 500 rpm for 5 min at 4°C for clarification, the supernatant fluids were frozen at -40°C overnight to reduce the nonbotulinic deaths of mice, and the sediments of the enrichment cultures were stored anaerobically at room temperature. After the samples were thawed at room temperature, a second centrifugation at 15,000 rpm for 15 min at 4°C clarified the supernatant fluids. The fluid from each of the 36 tubes, with and without trypsinization, was inoculated intraperitoneally into each of two 20- to 25-g ICR mice in 0.3-ml amounts. The inoculated animals were observed daily for 4 days. If both mice died with signs of botulism, neutralization of the remaining culture fluid with type-specific antitoxin (Centers for Disease Control, Atlanta, Ga.) was carried out. The sediments of toxic enrichment cultures were used for isolation of *C. botulinum* organisms by streaking these positive specimens on solid media (egg yolk agar) in triplicate for each specimen. The plates were incubated anaerobically at 35°C for 2 to 4 days. Each of the suspected colonies on the agar plates was tested for toxin production and for cultural and biochemical characteristics, following the procedures outlined by Dowell and Hawkins (5). The methods for detection of *C. botulinum* type G described in the next section and used in this study evolved from a series of comparative tests (Fig. 1).

ELISA for detection of type G toxin. Toxin and toxoid preparations, the immunization procedures (of two male

New Zealand White rabbits), the antitoxin titrations, and the immunoglobulin G purification of the collected serum specimens were performed by using techniques described previously (2, 11, 20). The ELISA method for the detection of type G toxin developed by Lewis et al. (11) was used without modification.

Supernatant fluids were collected from cultures of *C. botulinum* types A (NCTC 7272), B (NCIB 10642 and 10657), C (NCTC 8264 and 3732), D (NCTC 8265), E (NCIB 10660), F (NCIB 10641 and 10658), and G (isolates 89, 2739, and 2740), *C. subterminale* (isolates SG-155, SG-4495, M-11, and M-12), and *C. sporogenes* (isolates M-23 and M-35) grown in cooked-meat medium. These were clarified by centrifugation and examined by ELISA for specific identification of type G toxin and for interference and cross-reaction within this test system. In the initial experiments, the minimum amount of toxin detected by mouse assay, which was calculated by the method of Tint and Gillen (24), and the corresponding ELISA absorbance value were determined.

Gel immunodiffusion method for detection of type G strains. The procedure of Ferreira et al. (6) was adapted with two modifications. (i) *C. botulinum* type G antitoxin (Centers for Disease Control, Atlanta, Ga.) was incorporated into the gel diffusion agar overlay (1.2 to 1.5 IU/ml of gel diffusion agar). (ii) Tryptone-peptone-glucose-yeast extract agar and brain heart infusion agar at pH 7.6 were used as thin-layer agars.

Confirmation tests were performed with type G antitoxin, which was incorporated into gel diffusion agar overlays for different strains of *C. botulinum* types A through G, *C. subterminale*, *C. sporogenes*, *C. perfringens* (isolates SG-5672 and SG-6140), and *C. hastiforme* (isolates SG-2547 and SG-9147) for the specific detection of type G strains and for cross-reactions within this test system.

Incubated enrichment cultures in which type G toxin was identified by ELISA were tested by a gel immunodiffusion agar procedure for detection of type G strains. The sediment of each toxic enrichment culture diluted 1:10 in gelatin-phosphate buffer was used for isolation of type G organisms by evenly spreading 0.01 ml on each of the three plates with the two media (tryptone-peptone-glucose-yeast extract agar and brain heart infusion agar). After 48 h of anaerobic

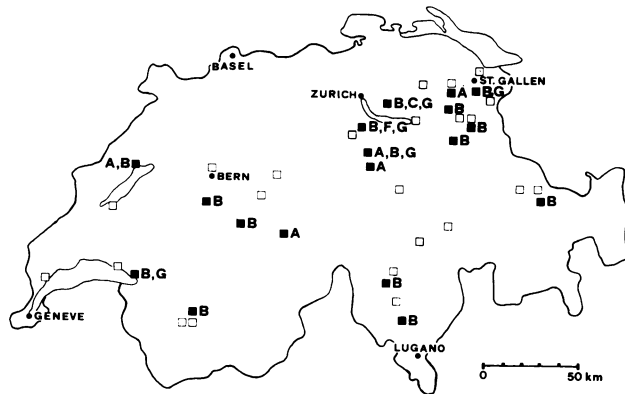


FIG. 2. Distribution of *C. botulinum* in soil samples of Switzerland. Shown are locations of samples where *C. botulinum* organisms were present (■) or absent (□); the letters A, B, C, F, and G are the toxin types of *C. botulinum*.

incubation at 35°C, the plates were overlaid with gel diffusion agar containing antitoxin of type G for 24 to 72 h at 25°C. Isolated colonies which were surrounded by zones of precipitation were picked for identification by mouse toxicity and neutralization tests and by biochemical characteristics.

Toxin production. The isolated strains of type G from the soil samples and the formerly isolated strains 89 G from Argentina and 2739 G and 2740 G from Switzerland were grown at 30°C. A seven-day-old culture of each strain in cooked-meat medium was centrifuged at 15,000 rpm for 15 min at 4°C, and the supernatant was filtered through a 0.45- μ m-pore-size membrane filter (Millipore Corp., Bedford, Mass.). The recovered supernatant fluid was divided into two parts. One part was treated with trypsin, and the other was not. Both were titrated for toxicity in mice. Serial twofold dilutions were made in gelatin-phosphate buffer. One-half milliliter of each dilution was inoculated intraperitoneally into each of six 20-g mice (ICR). The mice were observed for 5 days, and the deaths were recorded. The toxin content (mouse 50% lethal dose [MLD₅₀]) per milliliter of the culture fluid was calculated by the method of Tint and Gillen (24). Additionally, culture fluids containing vegetative cells, lysed cells, spores, and toxin were treated with trypsin and centrifuged, and the recovered supernatants were then tested for toxicity in mice.

RESULTS AND DISCUSSION

C. botulinum was found in 18 (43.9%) of the 41 soil samples. No strain of *C. botulinum* of any type could be demonstrated in 23 soil specimens. It is apparent that soil does contain organisms that can interfere with the demonstration of *C. botulinum* types A, B, C, E, and F (1, 9, 10, 15, 18). Consequently, the absence of *C. botulinum* organisms from soil specimens can be accepted only if it has been shown that no factors are present that inhibit the growth of *C. botulinum* cells. No provision was made for any inhibiting factor in our study.

The soil of Switzerland contains *C. botulinum* of types A, B, C, F, and G, with type B being most common (Fig. 2).

C. botulinum type A strains were demonstrated in five specimens, frequently in soil specimens from grassy areas. They were found together with a type B strain in one specimen and with types B and G in one specimen.

C. botulinum type B strains were demonstrated in 15 specimens. In nine samples, type B was found alone. Almost

all samples of soil containing *C. botulinum* type B alone came from soils from the alpine region. This finding is in agreement with that of other investigators, who found that type B was most frequent in soil of alpine areas (13). Of four toxigenic cultures from cultivated land, we found type B together with type G in two specimens and with type G plus type C and with type G plus type F in one specimen each. Type B strains were isolated in 11 of the 15 toxigenic cultures. Five of these isolated strains were nonproteolytic and produced protoxin requiring activation by trypsin for full activity.

C. botulinum type C (alpha) was found together with types B and G in only one sample, taken from soil near a drainage ditch.

No strain of *C. botulinum* types D and E could be demonstrated in the soil specimens, the cause of which is unknown. Possibly the soil of this geographical area has never been contaminated with these toxin types of *C. botulinum*, or possibly the organisms are unable to survive in the soil of this region. The detection of *C. botulinum* in soil may be inversely related to the occurrence of organisms that inhibit its growth. Some strains of *C. perfringens* and *C. sporogenes* from soil are inhibitory for type A, B, C, and F strains of *C. botulinum*, but inhibition was not described for type D strains (15). However, *C. botulinum* type D organisms and neurotoxin were identified in necropsy specimens collected from three human cadavers in this area (22). Each of the deaths was associated with *C. botulinum* type D in adults and was described as sudden and unexplained.

The occurrence of type F strains of *C. botulinum* is often associated with soil that is moist or actually wet much of the time (17). *C. botulinum* type F, a proteolytic strain, was demonstrated together with types A and G in only one soil sample from a well-watered grassy field in close association with a lake shore.

C. botulinum type G was identified in five soil samples (Fig. 2) in close association with cultivated land. Two of the five specimens in which type G was found were taken from garden soil, two were from soil under grassy fields, and one was from a vegetable field (Table 1). Type G was found together with a type B strain, with types B and C, with types B and F, with types A and B, and with a nonproteolytic strain of type B, in different soil specimens.

The methods finally adopted for the detection of type G organisms and toxin in soil samples are summarized in Fig. 1. Testing smaller amounts of a sample by a dilution method in multiple cultures was more productive than a method examining large inocula was, and incubation of unheated cultures was unsatisfactory. The use of large amounts of inocula and unheated specimens reduced the incidence of positive findings because overgrowth of other bacteria not only masks the presence of type G, a low toxin producer, but also may inactivate botulinum toxin until examination and may suppress the growth of *C. botulinum* organisms during incubation of the specimen. Type G was found in only a low percentage of samples when the enrichment cultures were not treated with trypsin (Table 1). The relatively rare occurrence of type G in trypsin-treated supernatant fluids was demonstrated by the low incidence in the tubes of each soil specimen tested. Type G was identified in only eight tubes of the trypsin-treated fluids, which were from two different samplings at locations where the organisms were isolated with the higher levels of type G toxin (Table 2). The data show the difficulty of detecting type G in soil specimens by that procedure. A culture medium giving higher type G toxicity is desirable, since trypsinization did not generally increase the low toxin titers commonly obtained in enrich-

TABLE 1. Demonstration of *C. botulinum* type G by trypsin treatment, ELISA, and gel immunodiffusion

Sample no.	Location (canton)	Character of specimen	<i>C. botulinum</i> types isolated	No. of tubes in which:				
				Type G toxin was identified in supernatant fluid ^a			Type G strains were detected in sediment	
				Non-trypsin treated ^b	Trypsin treated ^b	ELISA ^b	Gel diffusion ^c	Toxicity and neutralization ^d
CH-2	St. Gallen (SG)	Garden soil	Types B and G	0	0	3	2	1
CH-16	Uster (ZH)	Grassy field	Types B, C, and G	0	0	2	2	1
CH-17	Zug (ZG)	Grassy field	Types B, F, and G	0	0	2	2	1
CH-18	Brunnen (SZ)	Vegetable field	Types A, B, and G	0	1	3	2	2
CH-35	Territet (VD)	Garden soil	Types B ^e and G	3 ^f	7	8	7	6

^a Number of tubes of each soil specimen tested, *n* = 36.
^b Number of tubes in which toxin of type G occurred.
^c Number of tubes in which type G colonies were identified by gel diffusion.
^d Number of tubes in which type G organisms were identified by mouse bioassay.
^e Nonproteolytic strain of type B.
^f Toxin of type G identified in tubes which were incubated for 5 days at 30°C.

ment cultures of samples being studied. Trypsin-containing medium could be more sensitive in detection of the organisms (12) or as a guide to complete toxin production.

In the initial experiments, type G toxin and antigen were detected readily by ELISA. Specific cross-reactivity was not observed with culture supernatants of *C. botulinum* types A through F and *C. sporogenes*. Nonspecific cross-reactions occurred with three of the four *C. subterminale* isolates tested. The response to *C. subterminale* is related to the almost analogous metabolic and physiological characteristics exhibited by this species and *C. botulinum* type G (4). The only demonstrable difference between *C. subterminale* and *C. botulinum* type G is the production of the toxin. Type G toxin was demonstrated by ELISA in 18 tubes of the five soil specimens (Table 1). *C. botulinum* type G strains, however, were identified in only 15 sediments of these enrichment cultures by the gel immunodiffusion method. *C. subterminale* strains were detected from the other three sediment cultures.

An evaluation of the gel immunodiffusion method with type G antitoxin was carried out for different strains of *C. botulinum* and other *Clostridium* species. Two concentric precipitation zones surrounding each colony of type G were clearly visible. One small or less-intense precipitin zone was detected around *C. subterminale* colonies, the nontoxic variant of *C. botulinum* type G. No precipitin zones were found with the tested strains of *C. botulinum* types A through F, *C. sporogenes*, *C. perfringens*, and *C. hasti forme*, an organism related to *C. botulinum* type G and *C. subterminale*.

Isolated colonies of the sediment cultures in which zones of precipitation were observed were picked to identify the

organisms by mouse bioassay and by biochemical characteristics. The intensities and sizes of the concentric precipitin zones surrounding the individual colonies were examined macroscopically through a hand lens. If the former standards were used, that colonies of *C. subterminale* had a single zone of precipitation, in contrast with the double zone obtained with type G colonies, the presence of type G strains was demonstrated from 15 tubes of these sediment cultures by the gel immunodiffusion method. *C. botulinum* type G strains, however, were only identified from 11 tubes of the sediment cultures by the mouse bioassay procedure; *C. subterminale* strains were detected in the other four sediment cultures (Table 1) which resembled *C. botulinum* type G in all physiological characteristics, except for toxicogenicity. Cultures of these four *C. subterminale* strains sometimes gave smaller-diameter or less-intensive precipitin zones or both, but always gave two concentric precipitation zones around the individual colonies by the gel immunodiffusion method. Supernatant fluids of these isolated *C. subterminale* strains, which were maintained in cooked-meat medium for 7 days at 30°C, were studied by the Ouchterlony gel diffusion technique against type G antitoxin. The supernatant fluids gave more than three distinct precipitin bands (Fig. 3a, wells 1 and 2), which showed identity reaction with the precipitation bands formed by the *C. botulinum* type G strains isolated from different soil locations (Fig. 3a, wells 3 through 5). In contrast, the supernatants of clinical isolates of *C.*

TABLE 2. Toxin titers of *C. botulinum* type G strains

Strain	Toxin titers (MLD ₅₀ /ml)		
	Non-trypsin-treated supernatant fluid	Trypsin-treated supernatant fluid	Trypsin-treated culture fluid
CH-2	12	96	650
CH-16	18	110	520
CH-17	42	280	1,800
CH-18	280	3,500	13,000
CH-35	96	880	2,500
89 G	64	560	4,300
2739 G	36	380	850
2740 G	24	320	980

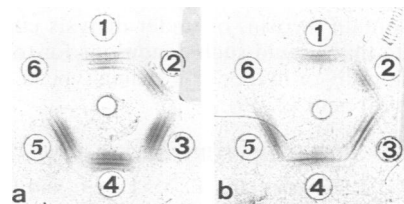


FIG. 3. Agar gel diffusion with precipitin bands formed by reacting type G antitoxin (center wells) against supernatant fluids in lateral wells. (a) Supernatants fluids of *C. subterminale* strains (isolates CH-18su [well 1] and CH-35su [well 2]), of *C. botulinum* type G strains (isolates CH-2 G [well 3], CH-18 G [well 4], and CH-35 G [well 5]), and of cooked-meat medium which was not inoculated but was incubated (well 6). (b) Supernatant fluids of different *C. subterminale* strains isolated from clinical specimens (isolates SG-3884 [well 1], SG-3885 [well 2], SG-51-4 [well 3], SG-4495 [well 4], and SG-155 [well 5]) and of cooked-meat medium which was not inoculated but was incubated (well 6).

subterminale gave only three precipitation bands (Fig. 3b, wells 1 through 5).

Various degrees of cross-reactivity within the heterogeneous group of clostridia collectively known as *C. botulinum* have been revealed. The results obtained with the ELISA and the electroblot procedures by Poxton (14) conclusively show that most strains of *C. botulinum* types A and proteolytic B (group I) share antigens with *C. sporogenes*, a nontoxic variant of *C. botulinum*, whereas types C and D (group III) share antigens with *C. novyi*. A type G (group IV) fluorescent-antibody reagent tested in a study by Glasby and Hatheway (8) successfully identified all seven strains of *C. botulinum* type G which were isolated from the two different locations but cross-reacted with two strains of *C. subterminale*. The relationship is similar to that of *C. sporogenes* of group I. Absorption of the type G fluorescent-antibody conjugate with *C. subterminale* removed activity for the Swiss strains but not for the two Argentinian strains. In view of the close taxonomic relationship between *C. botulinum* type G and *C. subterminale*, it appears that a type G antitoxin produced in immunologically tolerant animals could prevent the occurrence of cross-reactions within these different test systems, which was shown for *C. botulinum* type A in relationship to *C. sporogenes* by Dezfulian and Bartlett (3). Through induction of immunological tolerance to unwanted antigens of *C. sporogenes* and subsequent immunization with a relatively impure toxoid, they were able to produce a type A antitoxin for the detection of *C. botulinum* type A toxin by a sensitive ELISA without purifying the toxin.

The toxin titers produced by the isolated type G strains were relatively low (Table 2). The supernatants of type G cultures contained 12 to 280 MLD₅₀ per ml in media in which type A strains can produce 10,000 to 1,000,000 MLD₅₀ per ml. The toxin titers increased 10- to 100-fold after trypsin treatment of the supernatants. The titers of toxin produced in the same medium but with trypsin added after 7-days of incubation at 30°C to the whole culture fluids were potentiated 100- to 1,000-fold. Like the nonproteolytic strains of *C. botulinum*, toxin production is maximal at lower temperatures (26 and 30°C) in liquid media, and toxin is fully potentiated by trypsin (17, 19). Apparently, in instances of type G botulism, full toxicity may be attained by the action of proteolytic enzymes in the small intestine. In recent work (21) *C. botulinum* type G organisms were found in the ileocecal contents and type G toxin was demonstrated in the small bowel of two cases of sudden infant death syndrome; the toxin titers were 1,200 and 1,800 MLD₅₀ per g of feces. The higher toxin titers produced by this organism in media that contain trypsin, or under dialysis culture conditions, indicate that should such conditions for toxin production occur naturally or in food containing type G, this type of botulism could result (2, 19).

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