

Improved Medium for Sporulation of *Clostridium perfringens*¹

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An improved sporulation medium has been developed in which all five strains of *Clostridium perfringens* tested exhibited a 100- to 10,000-fold increase in numbers of spores when compared with spore yields in SEC medium under comparable conditions. In addition, three of five strains produced a 100- to 1,000-fold increase, with the remaining two strains yielding approximately the same numbers of spores, when compared with strains cultured in Ellner medium. At the 40-hr sampling time, 18 of 27 strains produced a 10- to 100-fold increase in numbers of spores in our medium, when compared to spore production obtained in a medium recently reported by Kim et al. The new medium contained yeast extract, 0.4%; proteose peptone, 1.5%; soluble starch, 0.4%; sodium thioglycolate, 0.1%; and $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 1.0%. In some cases, the spore yield could be increased by the addition of activated carbon to the new medium. The inclusion of activated carbon in the medium resulted in spores with slightly greater heat resistance than spores produced in the new medium without added carbon or in SEC or in Ellner medium. The major differences in heat resistance of the various strains appeared to be genetically determined rather than reflections of a particular sporulation medium. A definite heat-shock requirement was shown for four of four strains, with the optimal temperature ranging from 60 C for a heat-sensitive strain to 80 C for a heat-resistant strain. Heating for 20 min at the optimal temperature resulted in a 100-fold increase over the viable count obtained after heating for 20 min at 50 C.

It is very difficult to obtain a satisfactory degree of sporulation for many strains of *Clostridium perfringens*. Although attempts have been made to develop a medium in which a significant number of spores would be produced, only limited success has been achieved. The present study was undertaken with the objective of examining the effect of selected components within the medium on the degree of sporulation induced. It was hoped that this approach would result in a medium which would stimulate maximal sporulation. For comparison, the heat resistance of the spores produced in certain of the media was ascertained.

At present, the most abundant spore crops are usually obtained with Ellner medium (3), and this medium seems to be the one most commonly used. However, the SEC broth of Angelotti et al. (1), although permitting production of fewer spores than in Ellner medium, has been recom-

mended, especially for producing spores capable of surviving 100 C.

Kim et al. (10) have reported the development of a modified sporulation medium in which a larger number of spores was produced than in SEC broth. The spores were of greater heat resistance than those obtained in Ellner medium, when heating tests were performed using the Ellner medium as the suspending menstruum.

In a comparison of four media, it was previously confirmed in this laboratory (6) that the greatest number of spores was produced in Ellner medium. These spores, however, did not demonstrate the greatest stability with regard to heat resistance, and their morphology differed from those produced in the three other media.

MATERIALS AND METHODS

Strains. Five strains of *C. perfringens*, isolated from different sources, were selected for establishing optimal concentrations of medium ingredients. These strains were: American Type Culture Collection (ATCC) 3624 (classical type A); National Collection of Type Cultures (NCTC) 8238 (Hobbs' Type 2), isolated from boiled salt beef; NCTC 10240 (Hobbs'

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Type 13), isolated from chicken; 214a, isolated from beef and gravy by the Milwaukee, Wis., Health Department; and T-65, isolated in this laboratory from cooked turkey. All strains, except ATCC 3624, were associated with food-poisoning outbreaks. Once the sporulation medium had been developed, 22 other strains of *C. perfringens*, isolated from various meat, fecal, vegetable, and fly samples, were checked for their ability to sporulate in the new medium.

Stock cultures were maintained frozen in Cooked Meat Medium (Difco). Stock spore suspensions of the five different strains used in the initial studies were prepared in the basal medium to be described later. These spore suspensions were harvested from 24-hr cultures, washed three times with sterile, ice-cold water, and stored in distilled water at 4 C. These spore suspensions were used for subsequent studies.

Inoculation sequence. An active culture was obtained by inoculating 0.1 ml of the stock spore suspension into 10 ml of fluid Thioglycollate Medium (BBL) which was then heated at 75 C for 20 min and incubated at 37 C for 16 hr. Two subsequent transfers into 10 ml of fresh fluid Thioglycollate Medium, by use of a 10% inoculum and 4-hr incubation periods at 37 C, were then made. The entire contents of the last tube were inoculated into 100 ml of sporulation medium which had been freshly steamed and cooled to 37 C. The fluid Thioglycollate Medium was contained in 16 × 120-mm screw-cap tubes, whereas the sporulation medium was dispersed in 250-ml screw-cap prescription bottles. No attempts were made at anaerobic incubation other than the inclusion of sodium thioglycolate in the medium. When the time-course of growth and sporulation was followed, 2,500 ml of medium was used, with the inoculum being increased proportionally. The culture was agitated by means of a magnetic stirrer, with samples being taken through a serum-stoppered side spout. A water seal was used for gas evolution.

Sampling. A 5-ml sample of each sporulating culture was removed after 16 and 40 hr and was heated at 75 C for 20 min before plating in SPS agar (BBL). Dilutions were made in 0.1% peptone water, and incubation was carried out at 37 C and under a gas mixture of 90% nitrogen and 10% carbon dioxide. Viable counts were made after 48 hr.

Heating at 75 C for 20 min was recommended (5) as an appropriate time-temperature relationship for destroying vegetative cells of *C. perfringens*, thus allowing only spores to be determined by outgrowth in an agar medium. Per cent sporulation of an organism has often been determined on the basis of the number of colonies obtained before and after a given heat treatment. However, this method may be quite inaccurate for certain spore-forming organisms, because in some instances there is a heat-shock requirement for spores before they will undergo optimal germination and outgrowth.

It was noticed early that the total colony count of *C. perfringens* would sometimes be greater after heating at 75 C for 20 min than before such treatment. This suggested that the heat treatment was indeed stimulating additional germination and outgrowth of the spores. For this reason, the results presented here

are reported as the total numbers of viable spores produced, rather than as the percentage of total cells. A second reason for this procedure was that the low numbers of spores produced by many strains made it impossible to measure accurately per cent sporulation by direct microscopic counts.

Media. Of the two media currently recommended for sporulation of *C. perfringens*, neither produces very high numbers of spores, although Ellner medium is more effective than SEC medium. A basal medium was compounded utilizing some of the components present in Ellner and SEC media, in addition to lactose and L-arginine. Lactose was added because early studies with NCTC 8238 suggested that it was somewhat stimulatory to sporulation, whereas L-arginine was added because high concentrations have been shown to stimulate sporulation of *Clostridium botulinum* in a synthetic medium (12). The composition of the basal medium was: Trypticase, 2.0%; L-arginine, 0.25%; lactose, 0.1%; yeast extract, 0.2%; proteose peptone, 1.0%; soluble starch, 0.3%; sodium thioglycolate, 0.1%; and $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 0.5%. This basal medium was used for initial production of spores for further studies.

In modifying the basal medium, various components were omitted, one at a time, to determine their effect on sporulation of the five different strains. The components were also tested in different concentrations in order to determine the optimal concentration of those components found to be stimulatory to sporulation. If an ingredient was found to be nonstimulatory, it was omitted from the medium in the subsequent series of tests.

A great difference in numbers of spores produced was noted throughout these experiments, with the optimal concentrations of the various components of the medium varying considerably for each strain. In the final selection of the concentration of the individual components of the medium, the choice was usually made to favor increased sporulation in the strains which normally produced the lower numbers of spores.

Heat resistance studies. Heat resistance was measured by use of a constant temperature oil bath containing propylene glycol at 90 C. For heating, spores were put into Pyrex tubes 15 cm long with an outer diameter of 9 mm, and 3 ml of spore suspension was placed in each tube. The heating menstruum was 0.067 M phosphate buffer, pH 7.0. The sealed tubes were first placed in a 75 C water bath for 20 min, to allow for a heat-shock and to kill any vegetative cells. Immediately after heat-shocking, the tubes were placed in the 90 C bath. A come-up time of 1.23 min was determined with a Thermistor Probe (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio). At predetermined time intervals, duplicate tubes were removed from the bath and immediately cooled in ice water. Viable counts were determined by means of duplicate pour plates using SPS agar. Incubation was at 37 C for 48 hr, under a gas mixture of 90% nitrogen and 10% carbon dioxide.

Survivor curves were prepared by plotting the percentage of survivors against time on semilog paper, with each curve representing an average of two differ-

TABLE 1. Sporulation of five strains of *Clostridium perfringens* in four media^a

Strain	Ellner		SEC		Medium of Kim et al.		Proposed medium	
	Spores/ml × 10 ⁶		Spores/ml × 10 ⁶		Spores/ml × 10 ⁶		Spores/ml × 10 ⁶	
	16 hr	40 hr	16 hr	40 hr	16 hr	40 hr	16 hr	40 hr
ATCC 3624.....	11.0	6.3	.00023	.00014	0.54	0.48	4.75	7.87
NCTC 8238.....	0.067	0.088	.015	.00017	0.73	0.84	49.0	39.0
NCTC 10240.....	0.00007	0.00008	.00045	.0021	0.121	0.0004	0.086	0.07
214a.....	9.0	8.0	.083	.06	46.9	23.1	6.8	9.0
T-65.....	0.035	0.029	.00011	.00005	0.7	0.106	9.0	7.45

^a Ellner medium: polypeptone, 1.0%; yeast extract, 0.3%; starch, 0.3%; MgSO₄, 0.01%; Na₂HPO₄·7H₂O, 5.0%; KH₂PO₄, 0.15%; sodium thioglycolate, 0.01%. SEC medium: Trypticase, 2.0%; Casamino Acids, 2.0%; sodium thioglycolate, 0.1%; thiamine·HCl, 0.0001%. Medium of Kim et al.: peptone, 1.5%; Trypticase, 3.0%; starch 0.4%; NaCl, 0.5%; MgSO₄, 0.02%. Proposed medium: yeast extract, 0.4%; proteose peptone, 1.5%; starch, 0.4%; sodium thioglycolate, 0.1%; Na₂HPO₄·7H₂O, 1.0%. A 10% (by volume) inoculum of a 4-hr active culture was used in all cases.

ent determinations. The percentage of survivors was based on the initial number of spores producing outgrowth after heating at 75 C for 20 min.

RESULTS

Modification of the basal medium. Comparison of sporulation of five strains of *C. perfringens* in Ellner, SEC, and the basal medium, previously described in Materials and Methods, showed that in all cases sporulation was better in the basal medium than in SEC. However, only two of the strains (NCTC 8238 and 10240) sporulated better in the basal medium than in Ellner medium. In modifying the basal medium, L-arginine and lactose were omitted without appreciably increasing or decreasing the amount of sporulation obtained. The omission of starch, however, resulted in a greatly decreased turbidity of the cultures, with very little sporulation occurring. Increasing the starch concentration from 0.1 to 0.5% resulted in increased sporulation with all five strains, with 0.4 to 0.5% being the most effective.

Of various carbohydrates tested, dextrin was found to be capable of replacing the starch requirement in the sporulation medium. A concentration of 0.4% for both starch and dextrin was chosen for further testing in the basal medium.

Utilization of the two carbohydrates, starch and dextrin, by the organisms resulted in a drop in pH of the sporulation media. Therefore, varying the buffer concentration with fixed amounts of carbohydrate allowed a determination of the carbohydrate of choice for further studies. All strains but one produced higher numbers of spores in the presence of starch. Strain NCTC 10240 produced only slightly higher numbers with added dextrin. A concentration of 1.0% Na₂HPO₄·7H₂O was generally optimal with

both carbohydrates. Concentrations of buffer, approaching the 5.0% level used in Ellner medium, resulted in vegetative cells with abnormal morphology, as was often observed in Ellner medium.

Increasing concentrations of proteose peptone resulted in increasing numbers of spores, with 1.5% being selected as the concentration of choice. However, with the exception of strain 214a, the addition of Trypticase, in concentrations of 0.5 to 3.5%, resulted in the production of fewer spores. It has been reported (11) that the type of peptone used in a sporulation medium may be as critical as its concentration. Frequently, for many of the clostridia, Trypticase has not been found to be very good for inducing sporulation unless supplemented with animal tissue hydrolysates. The explanation for this phenomenon may be the possible absence of some factor or factors in the enzymatic casein hydrolysates.

The presence of yeast extract definitely increased sporulation in three of five strains. Little effect was noted with strain NCTC 10240, whereas a slight decrease in numbers of spores occurred with strain 214a. The greatest effect on sporulation was noted with NCTC 8238, which at concentrations of 0.4 to 0.5% produced triple the number of spores produced without added yeast extract. A concentration of 0.4% was selected for continuing studies.

The composition of the modified medium determined by the previously described experiments was: yeast extract, 0.4%; proteose peptone, 1.5%; soluble starch, 0.4%; sodium thioglycolate, 0.1%; and Na₂HPO₄·7H₂O, 1.0%. A comparison of sporulation in the five strains tested in Ellner medium, SEC medium, the medium of Kim et al., and in our proposed medium is presented in Table 1.

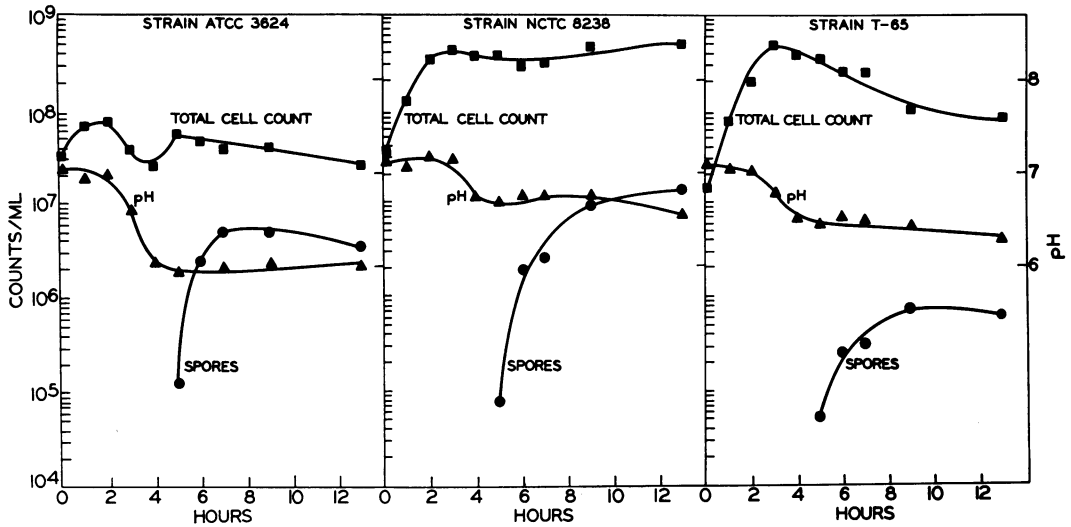


FIG. 1. Growth, sporulation, and pH change occurring during the culture cycle of three strains of *Clostridium perfringens* in the proposed sporulation medium.

TABLE 2. Effect of Norit A on sporulation of five strains of *Clostridium perfringens* in the proposed medium^a

Norit A (%)	ATCC 3624		NCTC 8238		NCTC 10240		214a		T-65	
	Spores/ml × 10 ⁶		Spores/ml × 10 ⁶		Spores/ml × 10 ⁶		Spores/ml × 10 ⁶		Spores/ml × 10 ⁶	
	16 hr	40 hr	16 hr	40 hr	16 hr	40 hr	16 hr	40 hr	16 hr	40 hr
0.0	0.895	0.145	36.0	22.0	.169	.081	7.4	6.1	14.5	3.7
0.05	1.35	0.08	139.0	175.0	.108	.0052	13.0	7.8	7.1	2.7
0.10	0.87	0.18	98.0	61.0	.295	.11	11.2	10.7	1.7	1.3
0.50	0.139	1.6	163.0	89.0	.425	.243	22.0	11.6	5.3	2.8
1.00	1.0	0.99	158.0	89.0	.78	.0012	27.0	18.5	6.0	1.6
1.50	0.44	0.83	123.0	113.0	.306	.003	24.0	11.9	5.5	3.6

^a See Table 1 for composition of proposed medium.

With the five strains tested, the modified medium yielded 100- to 10,000-fold increases over the numbers of spores produced in the SEC medium. Also, three of the five strains produced 100- to 1,000-fold increases in spore production over that of Ellner medium, with the remaining two yielding approximately the same numbers. The most recently reported medium for sporulation of *C. perfringens* is that of Kim et al. At the 40-hr sampling time, four of the five strains produced 10- to 100-fold increases in numbers of spores in the modified medium over that of the medium of Kim et al. A total of 18 of 27 different strains tested (strains listed in Table 3) also showed a 10- to 100-fold increase in numbers over that of the medium of Kim et al. The most dramatic increase was shown by strain NCTC 8238, presumably a food-poisoning strain with

paucity of spore production being one of its characteristics. In the proposed medium, 10⁷ spores/ml were obtained, as opposed to 10⁴/ml produced in Ellner medium and 10⁵/ml produced in the medium of Kim et al.

The time-course of growth, sporulation, and pH change, occurring during the culture cycle of three strains of *C. perfringens* in the proposed medium, is presented in Fig. 1. A drop in pH occurred with all strains between 2 and 4 hr of growth, just prior to the onset of sporulation. The pH was then maintained approximately constant throughout sporulation. Sporulation commenced between 4 and 5 hr with maximal populations being obtained quite rapidly. Strain ATCC 3624 reached a maximal spore population by 7 hr, whereas strains NCTC 8238 and T-65 reached a maximum by 10 to 12 hr. Recycling of the spores

TABLE 3. Sporulation of 27 different strains of *Clostridium perfringens* in proposed medium, with and without added carbon

Strain	Serological relationship to Hobbs' heat-resistant types	No. of spores/ml ($\times 10^6$) produced in proposed medium		No. of spores/ml ($\times 10^6$) produced in proposed medium plus 1.0% activated carbon grade NXC Mesh 6/8	
		16 hr	40 hr	16 hr	40 hr
NCTC 8238 ^a	2	49.0	39.0	148.0	124.0
NCTC 8239.....	3	0.12	0.025	1.25	0.4
NCTC 8678.....	5	0.07	0.04	0.27	0.75
NCTC 8679.....	6	0.0	0.0003	0.078	0.06
NCTC 8449.....	7	5.5	4.5	0.075	0.09
NCTC 8235.....	8	0.0	0.0	0.0	0.0
NCTC 8798.....	9	30.0	12.5	8.4	12.5
NCTC 9851.....	11	5.1	8.0	9.2	10.0
NCTC 10239.....	12	0.002	0.0015	0.01	0.012
NCTC 10240.....	13	0.086	0.07	0.00012	0.242
77455 ^b	10	0.0004	0.0006	0.0099	0.039
79393.....	10	0.09	0.08	0.01	0.014
77516.....	10	0.0002	0.0008	0.00026	0.012
79394.....	10	0.02	0.03	0.096	0.053
79955.....	10	0.0003	0.0006	0.0026	0.0023
F5a ^c	— ^l	0.0	0.0	0.0	0.0014
F12.....	—	0.83	0.03	0.69	1.48
027 ^d	—	0.027	0.00017	0.00012	0.272
021.....	—	0.018	0.0	0.00008	0.118
L83 ^e	—	0.0004	0.00036	0.00007	0.0029
L2.....	—	0.00007	0.00006	0.042	0.048
65 ^f	—	8.0	8.8	7.5	15.1
164 ^g	—	0.33	0.28	0.0046	0.061
127d ^h	—	0.048	0.05	3.2	5.7
214a ⁱ	—	6.8	9.0	38.0	25.1
T-65 ^j	—	9.0	7.45	0.0019	0.004
ATCC 3624 ^k	—	4.75	7.87	27.7	27.3

^a National Collection of Type Cultures, London, England.

^b Isolated from feces of patients suffering from food poisoning at University of Wisconsin.

^c Isolated from houseflies.

^d Isolated from bovine livers.

^e Isolated from market sliced beef liver.

^f Isolated from raw carrots.

^g Isolated from spiced sandwich loaf.

^h Isolated from raw ground beef.

ⁱ Isolated from beef and gravy by the Milwaukee, Wis., Health Department.

^j Isolated from turkey meat implicated in a food-poisoning outbreak in Madison, Wis.

^k American Type Culture Collection, Rockville, Md.

^l Serological relationship not determined.

of strain ATCC 3624 began to occur after 12 hr of growth.

Further attempts to improve the modified medium by the addition of various growth factors were unsuccessful. Although 10 vitamins, 7 minerals, 4 purines, and 4 pyrimidines were each tested at two different concentrations, none was beneficial in improving sporulation in the five strains studied.

Effect of activated carbon on sporulation. Activated carbon has been shown to increase the sporulation of such notoriously poor sporulators as *Bacillus popilliae* (9) and *B. larvae* (4), as well

as to improve the growth of fastidious microorganisms. The effect of Norit A, an activated carbon, on the sporulation of *C. perfringens* in the modified medium is shown in Table 2.

The addition of activated carbon resulted in a definite increase in sporulation with three of the five strains tested. No definite pattern of increase or decrease in sporulation was demonstrated with either strain ATCC 3624 or T-65. The most striking increase occurred with NCTC 8238 which increased from 10^7 to 10^8 spores/ml, this being the highest number of spores ever obtained with any of the strains tested. Approximately 1.0%

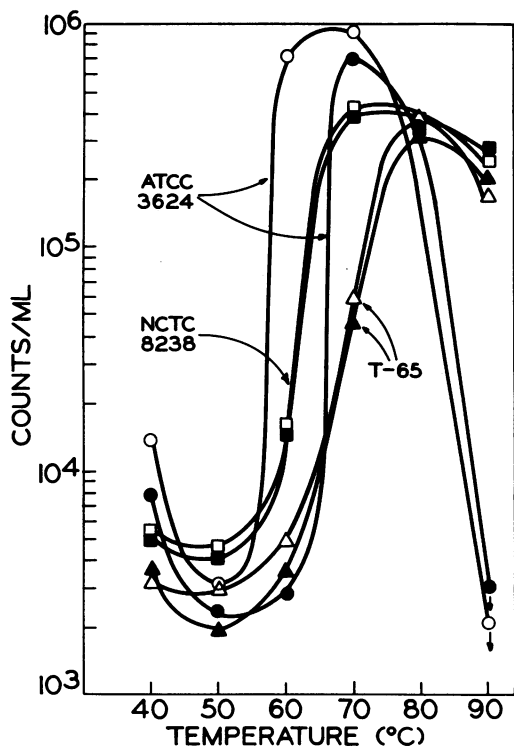


FIG. 2. Effect of heat-shock on spore germination and outgrowth. Spores were heated in 0.067 M phosphate buffer, pH 7.0. Symbols: open symbols, 20-min heating time; closed symbols, 10-min heating time; Δ , \blacktriangle , strain T-65; \square , \blacksquare , strain NCTC 8238; \circ , \bullet , ATCC 3624.

Norit A was judged to be the optimal concentration for the three strains.

An inherent problem in the incorporation of finely divided activated carbon in a sporulation medium is the subsequent separation of the spores from the carbon. Two grades of activated carbon, characterized by larger size particles, were obtained from the Union Carbide Corp. (New York, N.Y.), under the brand name "Columbia." The pelleted carbon was designated grade NXC Mesh 6/8, and the granular carbon was designated grade TS-570 Mesh 12/28. Addition of these carbons to the sporulation medium to a 1.0% concentration produced a response similar to that of Norit A, indicating that either could be substituted equally well for Norit A. A comparison of the sporulation of 27 different strains of *C. perfringens* in the modified medium, with and without the addition of 1% activated carbon grade NXC Mesh 6/8, is presented in Table 3.

At the 16-hr sampling time, 13 of the 27 strains showed an increase in the number of spores produced in the presence of added carbon, whereas,

at the 40-hr sampling time, 21 of the 27 strains showed an increase. Preliminary experiments have indicated that the carbon must be present in the medium during growth to increase sporulation. Extraction of the complete medium with carbon prior to sterilization produced decreased amounts of spores.

Effect of heat-shock on spore germination and outgrowth. Two heat-sensitive strains, ATCC 3624 and 214a, and two heat-resistant strains, NCTC 8238 and T-65, were heated at six different temperatures for 10 and 20 min time intervals. The spores were suspended in 0.067 M phosphate buffer, pH 7.0, for heating, and the viable spores were determined by plating in SPS agar.

A pronounced heat-shock requirement for optimal germination and outgrowth was evident for all four strains (Fig. 2). The optimal temperature for heat shock was approximately 70 to 75 C for NCTC 8238 and 80 C for T-65, with both 10 and 20-min heating times giving comparable results. Strain ATCC 3624 had an optimum of 60 to 70 C for the 20-min heating time and an optimum of 70 C for the 10-min heating time. The heat-shock requirements for strain 214a (not shown on graph) were comparable to strain ATCC 3624. The slight drop in counts obtained at 50 C may be due to inactivation of some vegetative cells present in the suspensions.

Heat resistance. The effect of heating at 90 C on the survival of spores of five different strains of *C. perfringens*, produced in Ellner, SEC, the medium of Kim et al., and our proposed medium, with and without added 1.0% activated carbon, is shown in Fig. 3.

Although some variation in the thermostability of the spores produced in different media exists, it is obvious that the major difference in the expressed heat resistance of different strains is genetically determined.

Two of the strains, NCTC 8238 and T-65, can be classed as heat resistant when compared with strains NCTC 10240, 214a, and ATCC 3624 (Fig. 3). Strains T-65, 214a, and ATCC 3624 produced the least heat-resistant spores in Ellner medium, but in SEC and in the modified medium, spores slightly higher and comparable in heat resistance were produced. In the medium of Kim et al., strain ATCC 3624 produced spores with the highest heat resistance of the four media, whereas spores of T-65 and 214a were comparable in heat resistance to those produced in the proposed medium and SEC medium. Strain NCTC 10240 when grown in Ellner, the medium of Kim et al., and the proposed medium produced spores of slightly greater heat resistance than in SEC, whereas strain NCTC 8238 produced spores of

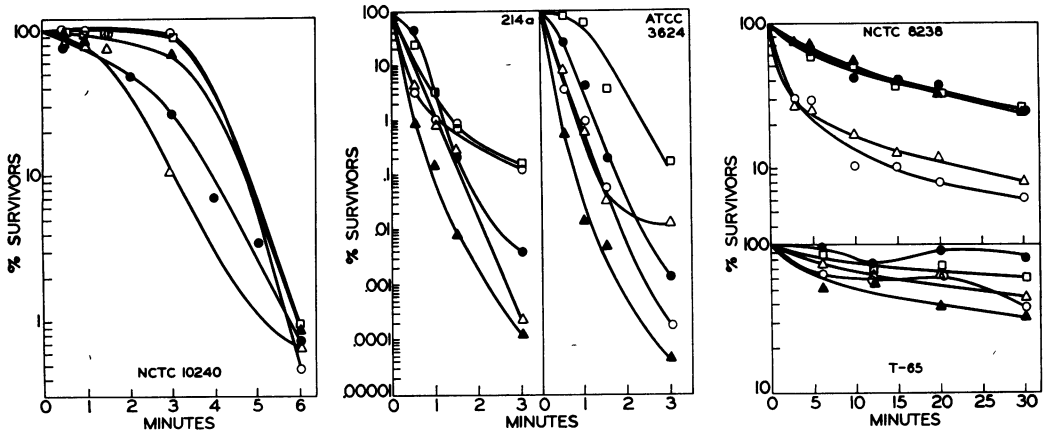


FIG. 3. Time-survivor curves showing the influence of sporulation media on spore heat resistance of *Clostridium perfringens* strains T-65, NCTC 8238, NCTC 10240, 214a, and ATCC 3624. Spores were heated in 0.067 M phosphate buffer, pH 7.0, at 90 C. Each point represents averages of duplicate trials at a given exposure time. Symbols: Δ , spores from SEC medium; \blacktriangle , spores from Ellner medium; \square , spores from the medium of Kim et al.; \circ , spores from the proposed medium; \bullet , spores from the proposed medium to which 1.0% activated carbon had been added.

greater resistance when grown in Ellner and in the medium of Kim et al., rather than in SEC or the proposed medium. When activated carbon was added to the proposed medium, four of the five strains produced spores which had an approximately equivalent or slightly greater heat resistance than the greatest resistance previously shown in the other media. Strain NCTC 10240 was slightly less heat-resistant when activated carbon was added to the medium.

DISCUSSION

C. perfringens is a notoriously poor sporulating organism. This is evident from the fact that, with many strains, spores can be demonstrated only by heating to destroy vegetative cells and then subculturing (7). Although *C. perfringens* has many growth requirements comparable to those of the more proteolytic clostridia (11), it has never been induced to sporulate to the degree that many of the proteolytic clostridia have.

The improved sporulation medium reported in this paper is a modification of a basal medium compounded from components present in both Ellner and SEC medium, the most commonly used media for sporulation of *C. perfringens*. In modifying the medium, concentrations and components were selected to favor increased sporulation of the poor sporulating strains. Although substantial increases in numbers of spores produced were obtained, the new medium has the drawback (found also in other media currently used) that high numbers of spores could not be produced with all strains of *C. perfringens* tested. The numbers of spores produced ranged from 0 with

strain 8235 to 10^8 spores/ml with NCTC 8238. This high number of spores was obtained only when activated carbon was added to the medium, a technique which also increased sporulation in 21 out of 27 strains tested.

Activated carbon has been shown to be partly responsible for the removal of antispore factors in complex organic media. Evidence has been presented (8) that these factors are inhibitory saturated fatty acids. In this study, the peptone used was an enzymatic digest of casein, which contained residual amounts of bound milk fat as the source of the inhibitory fatty acids. It was also suggested that the ability of cultures to generate free fatty acids during their growth may lead to suppressive action on the subsequent sporulation of the culture. The effect of activated carbon on sporulation of *C. perfringens* may be a reflection of the adsorption of such inhibitory fatty acids.

The inclusion of activated carbon in the proposed sporulation medium resulted in spores with slightly increased heat resistance over that of the proposed medium without added carbon and over the SEC and Ellner medium. Although SEC medium has been recommended (1) as the medium of choice in producing spores capable of surviving 100 C, the results reported here indicate that this ability is perhaps more dependent on the particular strain being used.

Weiss and Strong (*in press*) have shown that spores of *C. perfringens* produced in Ellner medium and suspended in the same medium for heating are much less heat resistant than those produced in SEC broth and suspended in SEC

