Evaluation of Media, Time and Temperature of Incubation, and Method of Enumeration of Several Strains of Clostridium perfringens Spores

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Received for publication 4 January 1974

Two basal media, containing the ingredients found in common in both SPS (BBL) and TSN (BBL) media and in the previously described media of Schaedler et al. (1965) and Starr et al. (1971), but minus antibiotics, were selected as the most suitable for the enumeration of Clostridium perfringens spores in a model system. These media were also used to study the influence of the presence of glucose, xylose, or ribose in various concentrations (0, 0.01, 0.1, and 1.0%) on colony morphology and spore recovery. As the sugar concentration in the basal agar medium increased, the colonies of all the test organisms also increased in size, and more of the black colonies became white in color. At the 1.0% sugar level, glucose permitted only white colony development, whereas the pentoses were completely inhibitory. Both pour plates and most-probable-number tubes were inoculated with the spores of several strains of C. perfringens and incubated at 20, 30, 37, and 45°C for 24, 48, and 72 h. Statistical analyses of the enumeration data indicated, at the 99% confidence level, that a Trypticase(BBL)-yeast extract-glucose-sulfite-iron agar gave maximal population estimates at 37°C in 72 h.

A variety of media and methods for the detection or enumeration, or both, of the genus Clostridium has been devised. Many of these media and procedures have been proposed for the isolation and quantitation of the pathogen Clostridium perfringens, a nonfastidious anaerobe capable of growing over a wide temperature range (15 to 50°C) (28). Gibbs and Freame (10), Mossel et al. (21), Smith (28), and Willis (33) have reviewed much of this literature. There are additional relevant contributions not included in these reviews (4–7, 11, 13, 16–18, 20, 21, 24, 25, 31). Comparisons of these qualitative and quantitative techniques have been made by many investigators (4–7, 10, 12, 13, 19, 21–23, 27, 29, 30, 32).

Because a number of the methods and media have been directed toward the selective isolation of C. perfringens from a mixed aerobic and anaerobic microflora, various chemical inhibitors and restrictive physical parameters (e.g., high incubation temperatures) have been utilized. Hence, preliminary evaluations in our laboratory for the selection of an appropriate enumeration method for C. perfringens spores in a model system resulted in considerable variations in the recovery rate and growth characteristics of the organism from strain to strain. Particular problems were noted in growth, colonial size and morphology, and the reduction of sulfite for forming blackened colonies. Our studies further indicated that monosaccharide supplementation of the media used and the manipulation of incubation parameters could significantly alter the growth pattern and characteristics of the organism.

Media such as SPS (2) (BBL), TSN (19) (BBL), and modifications of that of Schaedler et al. (25, 30) are relatively popular for the selective detection and enumeration of C. perfringens. We therefore selected (omitting all obvious inhibitors) the basal ingredients representing the first two media as our first test medium, and those of the last two media as our second test medium, for comparative study. Because it was deemed desirable that the enumeration medium, if possible, should encourage blackening of the cultures to obtain tentative identification of the test organism, we retained the sodium sulfite and ferric citrate in our first test medium. Each medium was then modified.

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with several concentrations of different mono-

saccharides, both as an agar and as a broth, for

use under various time and temperature condi-

tonics of incubation. A statistical analysis was

performed to determine the best parameters for

maximal recovery of the majority of the strains
tested.

MATERIALS AND METHODS

Test organisms. Strains of C. perfringens (NCTC 8238, NCTC 8798, NCTC 9851, NCTC 10240, FD-1, FD-2, FD-5, FD-7, T-65, 68900, 77455, and 80535) were obtained from C. H. Duncan, University of Wisconsin, Madison. Four strains (FD-1, FD-2, FD-7, and 80535) were used to ascertain the effect of supplementing a basal medium with various concen-

trations of several monosaccharides on the recovery of their respective spores, whereas all 12 strains were used to evaluate different media, incubation parameters, and enumeration techniques. Stock cultures were grown in air-exhausted (100 C for 15 min and cooled to 20 to 30 C) Cooked Meat medium (Difco) fortified with 0.1% glucose contained in screw capped tubes (20 by 150 mm), incubated at 37 C for 48 h, and stored at 2 to 5 C.

Spore suspensions. Stock spore suspensions were prepared biphasically (3) and kept at 2 to 5 C until required. Just before use, spore samples were heat-

shocked (65 C for 30 min) and diluted with chilled distilled water to yield test samples with about equal numbers of spores of all strains used.

Enumeration media. Two basal media were used. (i) The ingredients of TYE broth included the formula-
lations of both SPS (2) and TSN (19) media, modified as follows: 1.5% Trypticase (BBL), 1.0% yeast extract (BBL), 0.05% sodium sulfite, and 0.05% ferric citrate; the agar medium incorporated 1.5% plain agar (Fisher Scientific Co.). The medium was adjusted to pH 7.2 with 5 N KOH. (ii) SCH agar was a modification of the medium of Schaedler et al. (25) and of Starr et al. (30): 1.0% Trypticase (BBL), 0.5% polypeptide peptone (BBL), 0.5% yeast extract (BBL), 0.3% tri(hy-
droxymethyl)aminomethane, 0.001% hemin, 0.04% 1-cystine, and 1.35% plain agar (Fisher). This me-
dium was adjusted to pH 7.6 with 5 N KOH. Both media were supplemented with reagent grade glucose, ribose, or xylose in the dry form, in concentrations of 0, 0.01, 0.1 or 1.0% prior to autoclaving. The agar media were sterilized in 300-ml quantities, and the broth was sterilized in 20-ml volumes in screw capped tubes (20 by 150 mm), at 121 C for 15 min.

Plating procedure. Test spore suspensions were
diluted to yield approximately 20 to 30 colony-form-
ing units per plate of TYE agar. Six replicate plates per variable were inoculated with 1.0 ml of each test spore suspension, mixed with about 20 ml of melted agar (45 ± 1 C), and incubated at 37 C for 24 h under 90% N2:10% CO2 in the modified painter’s pressure tank previously described (3). In addition, plate counts used to evaluate optimum time-temperature conditions (24, 48, and 72 h at 20, 30, 37, or 45 C) were incubated in Anaero jars (BBL) by using CO2:H2 GasPaks (BBL) to obtain anaerobiosis. Colonies on the six replicate plates were averaged.

Most-probable-number (MPN) procedure. Ten-

dil serial dilutions were used with 10 replicate tubes per dilution of TYE broth containing 0.1% glucose and 0.1% agar. Each tube was inoculated with 1.0 ml of the appropriate spore dilution. Except for steaming (100 C for 10 min) and cooling rapidly (20 to 30 C) just prior to inoculation, no additional precautions were taken to make the medium anoxic. Incubation was conducted at the time-temperature variables cited above. Based upon the number of tubes showing turbidity, MPN counts were estimated by employing the Fisher and Yates statistical tables (8).

Statistical treatment. All enumeration data were subjected to an analysis of variance and to Tukey’s Studentized Range test of multiple comparisons (26). A comparison of the plating and the MPN methods was made by the Student’s t test (26), by using the standard estimate of the degrees of freedom for unequal-sized samples with unknown, unequal-sized variances.

RESULTS

Effect of monosaccharides, concentrations, and strains on comparative plate counts. Tables 1 and 2 show the influence of enriching a basal medium (TYE agar) with different quan-
tities of various monosaccharides on the ma-
crocolony counts of four strains of C. perfringens spores. The analysis of variance (Table 1) demonstrated that there was a highly signifi-

Table 1. Analysis of variance of effect of

concentrations of monosaccharides in

Trypticase-yeast extract agar on plate counts of

strains of C. perfringens spores

| Variable | D.F. | Mean square | F-value* | Critical* |
|----------|------|-------------|----------|-----------|
| Main effects: | | | | |
| (i) Monosaccharides | 2 | 2.538 | 294.7 | 4.70 |
| (ii) Concentrations | 3 | 1.874 | 217.6 | 3.86 |
| (iii) Strains | 3 | 357.7 | 41.54 | 3.86 |
| Interactions: | | | | |
| (i) + (ii) | 6 | 729.1 | 84.67 | 2.88 |
| (i) + (iii) | 9 | 284.8 | 33.07 | 2.48 |
| (i) + (ii) + (iii) | 18 | 285.0 | 34.26 | 2.88 |
| Error: | | | 81.57 | 9.47 |

* Degrees of freedom.
* Significant at the 99% confidence interval.
* Based upon experimental data.
* Based upon statistical theory (26).
Table 2. Multiple comparisons of effect of concentrations of monosaccharides in Trypticase-yeast extract agar on plate counts of strains of C. perfringens spores

| Variable     | No. of plate counts | Average plate count ($\times 10^{-4}$) |
|--------------|---------------------|--------------------------------------|
| Monosaccharide: |                     |                                      |
| Glucose      | 96                  | 20.26                                |
| Ribose       | 96                  | 12.31                                |
| Xylose       | 96                  | 10.61                                |
| Concentration: |                     |                                      |
| 0.0%         | 72                  | 14.00                                |
| 0.01         | 72                  | 17.57                                |
| 0.10         | 72                  | 18.68                                |
| 1.0          | 72                  | 7.36                                 |
| Strain:      |                     |                                      |
| FD-1         | 72                  | 15.35                                |
| FD-2         | 72                  | 15.75                                |
| FD-7         | 72                  | 15.44                                |
| 80535        | 72                  | 11.07                                |

a Plates were incubated at 37°C for 24 h under a 90% N₂; 10% CO₂ atmosphere.

The least significant difference (contrast) between average plate counts at the (1-α) level according to Tukey’s Studentized Range test of multiple comparisons.

Six plates per strain x four strains x one monosaccharide x four concentrations.

Six plates per strain x four strains x one monosaccharide x one concentration.

Six plates per strain x one strain x three monosaccharides x four concentrations.

Table 3. Colony characteristics of strains of C. perfringens on Trypticase-yeast extract agar supplemented with monosaccharides at various concentrations

| Strain | Monosaccharide | Concentration |
|--------|---------------|---------------|
|        | 0.00% | 0.01% | 0.10% | 1.0% |
| FD-1   | Glucose | SB    | MB    | MW-B  | LWG  |
|        | Ribose  | SB    | MB    | MW    | #    |
|        | Xylose  | SB    | MB    | MW-B  | #    |
| FD-2   | Glucose | SB    | MB    | MW-B  | LWG  |
|        | Ribose  | SB    | MB    | MW    | #    |
|        | Xylose  | SB    | MB    | MW-B  | #    |
| FD-7   | Glucose | SB    | MB    | MW-B  | LWG  |
|        | Ribose  | SB    | MB    | MW    | #    |
|        | Xylose  | SB    | MB    | MW-B  | #    |
| 80535  | Glucose | SB    | MB    | MW    | LWG  |
|        | Ribose  | SB    | MB    | MW    | #    |
|        | Xylose  | SB    | MB    | MW-B  | #    |

S: small, pinpoint colony; M: medium colony, 1 to 3 mm in diameter; L: large colony, 3+ mm in diameter; B: black; W: white; G: gassing with splitting of the agar. Plates were incubated at 37°C for 24 h under a 90% N₂; 10% CO₂ atmosphere.

No growth at this concentration.
perfringens. When TYE agar was supplemented with 1.0% monosaccharide, only the medium containing glucose permitted colony development in 24 h at 37 C. All the colonies were large (>3 mm in diameter) and white and produced gassing and splitting of the agar, so that enumeration was difficult. One percent xylose or ribose was completely inhibitory under our conditions.

Effect of media supplemented with 0.1% monosaccharides on comparative plate counts. Based upon the above information, additional enumeration studies were performed with basal agar media (TYE, SCH) containing 0.1% of the above sugars; this time 12 strains of C. perfringens were tested with each variable. Tables 4 and 5 statistically evaluate the influence of these factors, singly and in combination, on the plate counts. Again, all results were found to be highly significant and very large, even at the 99% confidence interval.

The largest main effect on plate counts was caused by the media used, followed by strain variation and monosaccharides, in that order (Table 4). Among the interactions, media-strains produced the most significant effect. In fact, this combination of factors yielded a higher F-value than the main effect due to the sugars.

Multiple comparisons were made of all the variables used in order to assess their detailed effects on the plate counts (Table 5). The sugar-fortified TYE agar produced twice as many colonies as the sugar-supplemented SCH agar. Tukey's test showed that, of the three monosaccharides used, glucose enrichment of the basal media resulted in the highest colony counts. The presence of either one of the pentoses in the media was inhibitory to the test organisms, because the plate counts were definitely lower than in the unfortified agar controls. Although the media containing these two sugars generated similar counts at the 99% confidence level, xylose supplementation gave significantly higher counts at the 95% level than ribose addition.

### Table 4. Analysis of variance of the effect of media supplemented with 0.1% monosaccharides on the colony counts of strains of C. perfringens spores

| Variable | D.F. | Mean square | F-value | Computed* | Critical* |
|----------|------|-------------|---------|-----------|-----------|
| Main effects: | | | | | |
| (i) Media | 1 | 56,803 | 4,393 | 6.68 | |
| (ii) Monosaccharides | 3 | 5,039 | 358 | 3.82 | |
| (iii) Strains | 11 | 10,452 | 743 | 2.29 | |
| Interactions: | | | | | |
| (i) + (ii) | 3 | 671 | 48 | 3.82 | |
| (i) + (iii) | 11 | 6,755 | 480 | 2.29 | |
| (ii) + (iii) | 33 | 714 | 51 | 1.71 | |
| (i) + (ii) + (iii) | 33 | 281 | 20 | 1.71 | |
| Error: | 480 | 14.07 | | |

* Media used were TYE and SCH agars (see Materials and Methods for composition).
* Degrees of freedom.
* Significant at the 99% confidence interval.
* Based upon experimental data.
* Based upon statistical theory (26).

### Table 5. Multiple comparisons of effect of media supplemented with 0.1% monosaccharides on the colony counts of strains of C. perfringens spores

| Variable | No. of plate counts | Average plate count (× 10^-4) | Δ_{95} |
|----------|---------------------|-------------------------------|-------|
| Medium: | | | |
| TYE | 288* | 39.74 | |
| SCH | 288 | 19.88 | 0.81 |
| Monosaccharide: | | | |
| None | 144* | 30.23 | |
| Glucose | 144 | 37.99 | |
| Ribose | 144 | 24.87 | |
| Xylose | 144 | 26.14 | 1.38 |
| Strains: | | | |
| NCTC 8238 | 48* | 30.02 | |
| NCTC 8798 | 48 | 23.67 | |
| NCTC 9851 | 48 | 39.04 | |
| NCTC 10240 | 48 | 19.02 | |
| FD-1 | 48 | 17.23 | |
| FD-2 | 48 | 32.92 | |
| FD-5 | 48 | 42.12 | |
| FD-7 | 48 | 23.62 | |
| T-65 | 48 | 68.42 | |
| 77455 | 48 | 22.42 | |
| 80535 | 48 | 24.35 | |
| Overall avg | | | 29.81 |

* Plates were incubated at 37 C for 24 h under a 90% N_2:10% CO_2 atmosphere.
* Δ_{95} is the least significant difference (contrast) between average plate counts at the (1-α) level according to Tukey's Studentized Range test of multiple comparisons.
* Six plates per strain × 12 strains × one medium × four monosaccharides × one concentration.
* Six plates per strain × 12 strains × two media × one monosaccharide × one concentration.
* Six plates per strain × one strain × two media × four monosaccharides × one concentration.
Among the strains examined, T-65 produced the highest colony counts, more than twice the overall average of all 12 strains and some five times the poorest growing strain (77455).

Among the remaining strains, FD-5, NCTC 9851, and FD-2, in that order, also yielded higher than average plate counts. Strain NCTC 8238 attained the overall average, but the remainder of the six organisms were below the average.

**Effect of incubation time and temperature and strains on comparative counts by the plating and MPN procedures.** To optimize the enumeration technique, incubation time-temperature studies were conducted by using colony and MPN counts on all 12 strains of *C. perfringens*. The medium of choice was TYE with 0.1% glucose (TYEG).

None of the strains formed colonies on plates or turbidity in the MPN tubes at 20 C in 24 h at the lowest dilution used, whereas six strains (NCTC 8798, NCTC 9851, FD-5, FD-7, T-65, and 80535) did not grow in the tubes within 48 h at this temperature, although they did form colonies on the plates under these conditions. At the highest dilution used, four strains (NCTC 8238, FD-2, FD-5, and FD-7) produced uncountable plates in 72 h at 30, 37, and 45 C, due to excessive gassing, splitting of the agar, blackening, and/or too many colonies per plate. Two strains (T-65 and 68900) caused a similar problem within 72 h at 37 and 45 C, two strains (FD-7 and 68900) within 48 h at 45 C, and strain FD-7 within 48 h at 37 C.

For purposes of statistical analysis, the numbers of organisms in all these cases were estimated. In the first instance, when a plate count of zero was obtained in all six replicate plates at the lowest (10⁻¹⁰) dilution used, the number of colonies was estimated by applying the binomial theorem and assuming that the probability of no colonies occurring in six trials is 0.5. The estimated colony count is therefore 0.08 x 10⁶ (see Appendix for details). In the situation where plates could not be read satisfactorily at a given temperature, the colony count at the next lower time interval at said temperature was used. In the case of no growth occurring in the MPN tubes for all dilutions used (10⁻¹⁰ to 10⁻⁴⁺ₓ) where x > 0 is a positive integer, the number of cells was estimated to be (n - 1.3), which is based on the assumption that at dilution 10⁻⁴⁺ₓ a maximum of four to five turbid tubes would have been found (see Appendix for details).

Tables 6 and 7 show the statistical analysis of the effect of time and temperature of incubation and strain variation on the two enumeration methods. This time, the counts were converted to logarithms to the base 10, because the variances of some subsets of the original data were too different to permit the correct application of the analysis of variance. Expressing the data as logarithms is an accepted method of handling this problem. All results were again found to be significant at the 99% confidence interval.

With the plate count method, the largest

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**TABLE 6. Analysis of variance of the effect of time, temperature, and method of enumeration of strains of *C. perfringens* spores**

| Variable | Plate count method¹ | MPN method² |
|----------|---------------------|-------------|
|          | D.F.¹ | Mean square | Computed¹ | Critical¹ |
|          |       |            |           |           |
| Main effects: |       |            |           |           |
| (i) Incubation time | 2     | 42.68      | 2,128     | 4.64      |
| (ii) Incubation temp | 3     | 57.53      | 2,868     | 3.81      |
| (iii) Strains | 11    | 88.95      | 4,434     | 2.28      |
| Interactions: |       |            |           |           |
| (i) + (ii) | 6     | 38.01      | 1,995     | 2.83      |
| (i) + (iii) | 22    | 0.2213     | 11.04     | 1.87      |
| (ii) + (iii) | 33    | 0.3985     | 19.87     | 1.70      |
| (i) + (ii) + (iii) | 66    | 0.3516     | 17.53     | 1.48      |
| Error:    | 720   | 0.02006    |           |           |

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¹ Agar plates (TYE + 0.1% glucose) were incubated at 20, 30, 37 and 45 C up to 72 h under a 90% N₂; 10% CO₂ atmosphere.

² Degrees of freedom.

³ Significant at the 99% confidence interval.

⁴ Based upon experimental data.

⁵ Based upon statistical theory (26).

⁶ Used 10 replicate tubes per dilution of TYE broth containing 0.1% glucose and 0.1% agar and incubated at 20, 30, 37 and 45 C up to 72 h.

⁷ Not estimable.
effect was caused by strain variation followed next by incubation temperature and then by time (Table 6). Among the interactions, time and temperature, as expected, yielded the greatest effect. With the MPN procedure, temperature and strain variation gave essentially identical effects (the comparative ratios of the F-values were 62.8 and 61.7, respectively), whereas time was the least influential. Again, incubation time and temperature was the major interaction.

Multiple comparisons of all the variables used are detailed in Table 7. With the plate count method, Tukey's test showed that the number of colonies increased with time, but the change was much smaller between 48 and 72 h than between 24 and 48 h. The same pattern occurred with temperatures from 20 through 45 C. Strain 68900 produced the highest number of colonies, followed closely by FD-1, T-65, and NCTC 8238; the lowest colony counts occurred with strains 77455 and 80535. Six strains yielded counts above the overall average, three strains were below, and the remaining

TABLE 7. Multiple comparisons of the effect of time, temperature, and method of enumeration of strains of C. perfringens spores

| Variable | Plate count method* | MPN method* |
|----------|---------------------|-------------|
|          | No. of plate counts | Avg plate count (log,0) | Δₘ₉₉ | No. of tube counts | Avg MPN count (log,0) | Δₘ₉₉ | α₀.₉₅ |
| Time (h): |                     |                       |       |                        |                         |       |       |
| 24       | 288*                | 5.84                 | 0.034 | 48'                     | 5.46                    | 0.181 | 0.42  |
| 48       | 288                 | 6.46                 | 0.034 | 48                      | 5.88                    | 0.181 | 0.42  |
| 72       | 288                 | 6.55                 | 0.034 | 48                      | 5.95                    |       |       |
| Temp (C):|                     |                       |       |                        |                         |       |       |
| 20       | 216*                | 5.51                 | 0.037 | 36*                     | 4.62                    | 0.224 | 0.38  |
| 30       | 216                 | 6.48                 | 0.037 | 36                      | 5.89                    | 0.224 | 0.38  |
| 37       | 216                 | 6.57                 | 0.037 | 36                      | 6.34                    | 0.224 | 0.38  |
| 45       | 216                 | 6.56                 | 0.037 | 36                      | 6.21                    | 0.224 | 0.38  |
| Strains: |                     |                       |       |                        |                         |       |       |
| NCTC 8238| 72*                 | 7.11                 | 0.044 | 12*                    | 6.66                    | 0.406 | 0.52  |
| NCTC 8798| 72                 | 6.33                 | 0.044 | 12                     | 5.64                    | 0.406 | 0.52  |
| NCTC 9851| 72                 | 6.30                 | 0.044 | 12                     | 5.78                    | 0.406 | 0.52  |
| NCTC 10240| 72                | 5.28                 | 0.044 | 12                     | 4.91                    | 0.406 | 0.52  |
| FD-1     | 72                  | 7.25                 | 0.044 | 12                     | 7.39                    | 0.406 | 0.52  |
| FD-2     | 72                  | 7.03                 | 0.044 | 12                     | 6.59                    | 0.406 | 0.52  |
| FD-5     | 72                  | 6.33                 | 0.044 | 12                     | 5.08                    | 0.406 | 0.52  |
| FD-7     | 72                  | 6.67                 | 0.044 | 12                     | 5.76                    | 0.406 | 0.52  |
| T-65     | 72                  | 7.24                 | 0.044 | 12                     | 6.26                    | 0.406 | 0.52  |
| 68900    | 72                  | 7.35                 | 0.044 | 12                     | 6.90                    | 0.406 | 0.52  |
| 77455    | 72                  | 4.26                 | 0.044 | 12                     | 4.09                    | 0.406 | 0.52  |
| 80535    | 72                  | 4.26                 | 0.044 | 12                     | 4.13                    | 0.406 | 0.52  |
| Overall avg | 6.31            | 5.77                |        |                         |                         |       | 0.23  |

* Plates were incubated under a 90% N₂:10% CO₂ atmosphere by using TYEG agar.
* Ten replicate tubes per dilution of air-exhausted TYEG broth containing 0.1% agar were used.
* Δₘ₉₉ is the least significant difference (contrast) at the (1-α) level according to Tukey's Studentized Range test of multiple comparisons within each method.
* Least significant difference between the plating and MPN method at the 95% confidence interval by the Student's t test.
* Six plates per strain × one incubation time × four incubation temperatures × 12 strains.
* One reading per strain (time and temperature) × one incubation time × four incubation temperatures × 12 strains.
* Six plates per strain × three incubation times × one incubation temperature × 12 strains.
* One reading per strain (time and temperature) × three incubation times × one incubation temperature × 12 strains.
* Six plates per strain × three incubation times × four incubation temperatures × one strain.
* One reading per strain (time and temperature) × three incubation times × four incubation temperatures × one strain.
organisms attained the average.

By using the MPN procedure, Tukey's test again disclosed that growth was lowest in 24 h and that 48 and 72 h gave higher but essentially the same results. Incubation at 37 and 45 C produced the highest but similar estimates, 30 C yielded significantly lower counts, and 20 C gave the lowest MPN. In fact, estimated counts at 20 C were less than 0.1 of those obtained at 30 C or above. Strain FD-1 produced the highest MPN, followed by 68900, NCTC 8238, and FD-2, which did not differ significantly from each other. The poorest growing strains were 77455 and 80535, which yielded about the same estimated counts and were some three orders of magnitude less than those for FD-1. Five strains gave counts above 1, five strains were below, the overall average MPN, whereas two strains attained the mean.

**Comparative counts between the plating and MPN procedures.** The agreement between the counts obtained by the plating and MPN methods was not very close (Table 7). In 10 of the 19 comparisons (time, temperature, and strains), the two procedures gave significantly different estimates at the 95% confidence interval. The overall logarithmic mean for the colony counts (6.31) and the MPN estimates (5.77) were also significantly different at the 95% level, the least significant difference (δa.s.s) being 0.23. Hence, the logarithms of the plate counts exceeded those of the MPNs by 0.54, indicating that the former method gave about 3.5-fold higher spore numbers than the latter technique.

Regardless of the enumeration procedure used, the rank (numerical order) of the spore concentrations estimated for the 12 strains, from highest to lowest, is statistically similar (Table 8). This is indicated by the rank-correlation coefficient expressed as \( r_s = 1 - \frac{6}{N(N^2 - 1)} \sum_{i=1}^{N} (R_{pi} - R_{pi})^2 \), where \( N \) is the number of strains (12) and \( R_{pi} \) and \( R_{pi} \) are the ranks according to the plating and MPN techniques of the \( i \)-th strains. If \( R_{pi} = R_{pi} \) for each \( i \), then \( r_s = 1 \), but if \( r_s \) is near zero then the results of the two methods are unrelated. The data in Table 8 compute to an \( r_s = 0.923 \), indicating that the results are rather closely related at the 99% confidence level.

**Principal statistical conclusions.** Ribose and xylose inhibited the growth of *C. perfringens* spores, but glucose enhanced it, the greatest increase being with 0.01 and 0.1% concentrations. The TYEG basal medium produced much more growth than the SCH basal medium. TYE with 0.1% glucose (TYEG) produced the highest number of colonies in 72 h at 37 C. The two methods of enumeration (plate count and MPN) gave qualitatively similar results with the variables tested, but the plating method gave higher numerical values with 11 of the 12 strains studied.

**DISCUSSION**

*C. perfringens* can be successfully propagated on a wide variety of laboratory media under anaerobic conditions. However, egg yolk media (11, 17, 27, 34, 35) were omitted from this study due to problems of opacity, which rendered them somewhat unsuitable for pour plate and broth cultivation. The deep agar tube-counting technique was eliminated relatively early from our experiments due to severe gassing and excessive splitting and blackening of the agar with nearly all media examined. Also, incubation of MPN tubes was not permitted to exceed 72 h for practical reasons. Under our experimental conditions, the Trypticase-yeast extract-glucose-sulfite-iron agar (TYEG) pour plate gave maximal population estimates when spore samples were incubated at 37 C for 48 h for 11 of the 12 strains tested.

The selection of the monosaccharides used was not based solely on the ability of the organism to ferment them, because not all strains actively fermented ribose and xylose. Preliminary comparisons between glucose, lactose, maltose, mannose, and sucrose showed little variation in growth pattern and gassing ability when the organisms were cultured in basal broths supplemented with these compounds, whereas gassing was reduced in the presence of ribose or xylose without loss of growth enhancement. The latter two pentoses, however, were repressive to growth relative to glucose (Table 2) and were especially inhibitory.
at the 1.0% concentration, for the agar media containing these sugars gave significantly lower counts than the unfortified media (Table 5).

The apparent interaction between monosaccharides, incubation variables, and sulfite reduction would suggest a variation in redox potential according to the level and type of sugar present. Mead (20) has shown that colony blackening with hydrogen sulfide is redox-dependent and can be enhanced with various reducing agents, an agar overlay, or an extended period of incubation. With a capability to use both molecular hydrogen and a variety of internal enzyme donor sources, Fuchs and Bonde (9) have noted that rapid dehydrogenation of glucose by \textit{C. perfringens} will enhance hydrogen sulfide formation, particularly in the presence of nicotinamide. These findings further suggest that our agar-blackening problem was not only redox-related, but, also, that the monosaccharides tested had induced alternate metabolic pathways which had reduced the availability of H+ ions with increasing concentrations of the sugars. No measurements of redox potential-reducing mieties or nicotinamide were made in our investigations, nor did we attempt to overlay our agar plates with medium to determine whether blackening of any white colonies would reoccur by this technique. Studies to elucidate the role of the monosaccharides in the metabolism of \textit{C. perfringens} by manipulating the redox potential and the pH, among other things, are certainly indicated.

Regardless of the monosaccharides used, increasing concentrations produced larger but fewer numbers of black colonies with every strain tested (Table 3). At the 1.0% sugar level, only glucose permitted colony development, but none of the colonies were black.

Perhaps food products which had undergone some chemical degradation in storage due to food tissue and microbial activities might contain hydrolytic products such as ribose and/or glucose. If these foods are quantified for \textit{C. perfringens} in selective sulfite-containing media, could sufficient quantities of this sugar(s) be carried over in the inocula to (i) alter the appearance of the agar colonies and/or (ii) decrease the number of countable colonies, and hence result in a lower total count? This possibility may require examination.

Because the two pentoses at the 1.0% concentration were inhibitory to colony formation of \textit{C. perfringens} spores in a basal medium (Table 3), it would be of interest to examine the effect of these sugars on spore germination and outgrowth to determine where the inhibition occurred. Ando (1) studied the influence of both ribose and xylose on spore germination of \textit{Clostridium botulinum} type E in a minimal medium. He reported that in the presence of a 2 mM concentration of either sugar no germination took place in 60 min at 37 C. Increasing the ribose and xylose 10-fold initiated, respectively, only a 5 and 8% fall in optical density, and in the presence of a 100 mM (1.5%) concentration germination remained about the same with ribose and doubled (to 18%) with xylose. By using 0.03 M (0.45%) xylose, Heiglman et al. (14) observed only 7% germination of spores of \textit{Bacillus thermoacidurans} (coagulans) in a broth incubated for 6 h at 67 C and 30% germination in 48 h. Neither investigator reported on the influence of these pentoses on events following spore germination. Hyatt and Levinson (15) observed no germination effect of ribose or xylose on \textit{Bacillus megaterium} spores at a concentration of 25 mM (0.375%) in 2 h at 30 C, but both compounds did effectively promote subsequent postgerminative development. However, we are not aware of any spore germination or postgerminative studies performed on \textit{C. perfringens} with monosaccharides other than glucose.

**APPENDIX**

A. No colonies in agar plates at all dilutions used: The probability, \( P \), of obtaining zero colonies in six replicate plates at dilutions \( 10^{-n} \), \( 10^{-n+1} \), etc. is assumed to be \( P(0) = \frac{1}{2} \), and the probability of getting at least one colony on one plate is \( P \); hence, by the binominal distribution, \( P(0) = (1 - p)^n \) or \( \frac{1}{2} = (1 - p)^n \). Thus, \( p = 1 - e^{-n} \approx 0.1 \). The estimated colony count, therefore, is \( (1 \times 10^n) (0.8) - 0.08 \times 10^n \), where 0.8 is the initial dilution factor of the stock spore suspension prior to the preparation of the consecutive 10-fold test samples.

B. No growth in MPN tubes at all dilutions used: If no growth occurred at dilutions \( 10^{-n} \), \( 10^{-n+1} \), etc., we assume that at dilution \( 10^{-n+1} \) the number of positive tubes would have been four or five, the largest number that is consistent with obtaining zero positive tubes at the \( n \)-th dilution. The calculated number of spores, therefore, is either \( 0.436 \times 10^{-1} \) or 0.576 \( 10^{-1} \), respectively (26). Converting to \( \log_{10} \), we get \( n - 0.36 \) to \( n - 0.24 \) or roughly \( n - 1.3 \).

**ACKNOWLEDGMENTS**

We thank G. Silverman and W. Chesbro for critical review of this manuscript.

**LITERATURE CITED**

1. Ando, Y. 1971. The germination requirements of spores of \textit{Clostridium botulinum} type E. Jap. J. Microbiol. 15:515-525.
2. Angelotti, R., H. E. Hall, M. J. Poter, and K. H. Lewis. 1962. Quantitation of \textit{Clostridium perfringens} in foods. Appl. Microbiol. 16:199-199.
3. Clifford, W. J., and A. Anellis. 1971. \textit{Clostridium perfringens}. I. Sporulation in a biphasic glucose-ion-exchange resin medium. Appl. Microbiol. 22:856-864.
4. de Waart, J. 1971. The enumeration of clostridia in food. Arch. Lebensmittelhyg. 12:149-154.
5. de Waart, J., and H. Pouw. 1970. Studies on the suitability of blood-free media for the enumeration of clostridia. Zentralb. Bakteriol. Parasitenk. Infektionskr. Hyg. Abt. 1 Orig. 214:551-552.
6. de Waart, J., and F. Smitt. 1967. The enumeration of obligately anaerobic bacteria using pouches made from plastics with a low oxygen permeability. Lab. Pract. 16:1098-1099, 1105.

7. Drasar, B. S. 1967. Cultivation of anaerobic intestinal bacteria. J. Pathol. Bacteriol. 94:417-427.
8. Fisher, R. A., and F. Yates. 1953. Statistical tables for biological, agricultural and medical research, 4th ed. Hafner Press, New York, N.Y.
9. Fuchs, A.-R., and G. J. Bonde. 1957. The availability of sulphur for Clostridium perfringens and an examination of hydrogen sulphide production. J. Gen. Microbiol. 16:330-340.
10. Gibbs, B. M., and B. Freame. 1965. Methods for the recovery of clostridia from foods. J. Appl. Bacteriol. 28:95-111.
11. Hall, W. M., J. S. Witzeman, and R. Janes. 1969. The detection and enumeration of Clostridium perfringens in foods. J. Food Sci. 34:212-214.
12. Harmon, S. M., D. A. Kautter, and J. T. Peeler. 1971. Comparison of media for the enumeration of Clostridium perfringens. Appl. Microbiol. 21:922-927.
13. Harmon, S. M., D. A. Kautter, and J. T. Peeler. 1971. Improved medium for enumeration of Clostridium perfringens. Appl. Microbiol. 22:688-692.
14. Heiligman, F., N. W. Desrosiers, and H. Broumand. 1956. Spore germination. I. Activators. Food Res. 21:63-69.
15. Hyatt, M. T., and H. S. Levinson. 1964. Effect of sugars and other carbon compounds on germination and postgerminative development of Bacillus megaterium spores. J. Bacteriol. 88:1403-1415.
16. Lyons, C., and C. R. Owen. 1942. Wilson Blair medium in the rapid diagnosis of gas gangrene. J. Bacteriol. 43:685-687.
17. McClung, L. S., P. Heidenreich, and R. Toabe. 1946. A medium for the Nagler plate reactions for the identification of certain clostridia. J. Bacteriol. 51:751-752.
18. Macfarlane, R. G., C. L. Oakley, and C. C. Anderson. 1941. Haemolysis and the production of opalescence in serum and lecitio-vitellin by the toxin of Clostridium welchii. J. Pathol. Bacteriol. 52:96-100.
19. Marshall, R. S., J. F. Steenbergen, and L. S. McClung. 1965. Rapid technique for the enumeration of Clostridium perfringens. Appl. Microbiol. 13:559-563.
20. Mead, G. C. 1969. The use of sulphite-containing media in the isolation of Clostridium welchii. J. Appl. Bacteriol. 32:358-361.
21. Mosel, D. A. A., A. S. De Bruin, H. M. J. Van Diepen, C. M. A. Vendrig, and G. Zoutewelle. 1956. The enumeration of anaerobic bacteria, and of Clostridium species in particular, in foods. J. Appl. Bacteriol. 19:142-154.
22. Narayan, K. G. 1966. Selective agents in cultivation of clostridia. Zentralb. Bakteriol. Parasitenk. Infektionskr. Hyg. Abt. 1 Orig. 201:57-64.
23. Riemann, H. 1967. Recovery of anaerobic spores in different laboratory media, p. 75-79. In V. Fredette (ed.), The anaerobic bacteria. Montreal University, Montreal, Que., Canada.
24. Sasserman, A. T., Horodniceanu, E. Solomon, and P. Milosevic. 1961. Contributions to the study of the methods applied for the isolation of Clostridium perfringens. II. Isolation of Clostridium perfringens from food stuffs (sausages) by the enrichment technique at 46 C-47 C. Microbiol. Parazitol. Epidemiol. 6:421-424.
25. Schaedler, R. W., R. Dubos, and R. Costello. 1965. The development of the bacterial flora in the gastrointestinal tract of mice. J. Exp. Med. 122:59-66.
26. Scheffé, H. 1959. The analysis of variance. John Wiley and Sons, New York.
27. Shahidi, S. A., and A. R. Ferguson. 1971. New quantitative, qualitative, and confirmatory media for rapid analysis of food for Clostridium perfringens. Appl. Microbiol. 21:500-506.
28. Smith, L. D. 1972. Factors involved in the isolation of Clostridium perfringens. J. Milk Food Technol. 35:71-76.
29. Southworth, J. M. L., and D. H. Strong. 1964. Comparison of media for the isolation of Clostridium perfringens from food. J. Milk Food Technol. 27:205-209.
30. Starr, S. E., G. E. Killgore, and V. R. Dowell, Jr. 1971. Comparison of Schaedler agar and Trypticase soy yeast extract agar for the cultivation of anaerobic bacteria. Appl. Microbiol. 22:655-658.
31. Takacs, J. 1967. Methods for detection and isolation of clostridia in the complementary bacteriological meat and food examination, p. 101-113. In V. Fredette (ed.), The anaerobic bacteria. Montreal University, Montreal, Que., Canada.
32. Takacs, J. 1971. Comparative examinations for the effectiveness of media to detect clostridia from canned meat products. Arch. Lebensmittelhyg. 22:101-104.
33. Willis, A. T. 1965. Media for clostridia. Lab. Pract. 14:690-696.
34. Willis, A. T., and G. Hobbs. 1957. A modified Nagler medium. Nature (London) 180:92-93.
35. Willis, A. T., and G. Hobbs. 1969. Some new media for the isolation and identification of clostridia. J. Pathol. Bacteriol. 77:511-521.