1969

Physiological and taxonomic aspects of Clostridium thermocellum

James Gary Vidrine
Iowa State University

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VIDRINE, James Cary, 1936-
PHYSIOLOGICAL AND TAXONOMIC ASPECTS OF CLOSTRIDIUM THERMOCELLUM.

Iowa State University, Ph.D., 1969
Bacteriology

University Microfilms, Inc., Ann Arbor, Michigan
PHYSIOLOGICAL AND TAXONOMIC ASPECTS OF

CLOSTRIDIUM THERMOCELLUM

by

James Gary Vidrine

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY
Major Subject: Bacteriology

Approved:

Signature was redacted for privacy.

In Charge or Major Work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State University
Ames, Iowa

1969
PLEASE NOTE:
Several pages contain colored illustrations. Filmed in the best possible way.

UNIVERSITY MICROFILMS
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PART I. FURTHER STUDIES OF CLOSTRIDIUM THERMOCELLUM STRAIN C & E
INTRODUCTION

Campbell, in 1954, reported on an obligate thermophilic bacterium which was capable of growth at mesophilic temperatures when provided with certain exogenous nutritional growth factors. More recently (Ljunger, 1968), obligate thermophilic bacteria have been reported that grow at mesophilic temperatures when inhibitory substances are removed from the growth medium. But to the knowledge of the author the work of Oates et al. (1961, 1963, 1964) was the first known report of a mesophile being able to grow at elevated temperatures; however, recent work by Dowden et al. (1968) suggests that a mesophilic strain of *Bacillus subtilis* can be adapted to grow at elevated temperatures.

An interesting aspect of the symbiotic culture described by Oates et al (1961, 1963, 1964) was the so-called "thermophily factor", produced by the thermophile, which permitted the mesophilic symbiont to grow at 65°C. This project was initiated, therefore, to study this unique symbiotic relationship—particularly to study the nature of the "thermophily factor".
HISTORICAL

The area of microbiology concerned with cellulose-digesting bacteria is one which has long been plagued with considerable confusion and contradiction. Winogradsky (1929) working with Cytophaga, a group of mesophilic, cellulose-digesting, bacteria, believed that these organisms were unable to grow on soluble carbohydrates, but needed cellulose as the principal energy source.

Stanier (1942a, b) demonstrated that cytophaga were capable of fermenting monosaccharides if the sugars were filter-rather than heat-sterilized.

Hungate (1950) described several strains of a cellulolytic rumen bacterium which, although capable of fermenting cellulose and cellobiose, failed to ferment glucose, despite filter sterilization of this carbohydrate.

Studies dealing with thermophilic, cellulose-digesting bacteria were even more confused. It was reported (McBee, 1950) that Macfadyen and Blaxall (1894, 1896, 1899), working with enrichment culture techniques which employed as substrates mud, cotton and straw, were able to obtain actively fermentative cultures; they were unable, however, to purify their cultures, a circumstance which frequently resulted in the isolation of clones that failed to ferment cellulose.

A number of hypotheses for inability to isolate stable cellulose-digesting bacteria were advanced: Viljoen, Fred and
Peterson (1926), working with an anaerobic, cellulose-digesting thermophile, which they named Clostridium thermocellum, claimed that this culture irretrievably lost the ability to ferment cellulose after having been cultivated on media which lacked this carbon source.

Pringsheim (1912, 1913) believed that cellulose-digesters were unable to grow in agar, a condition which was later refuted by Tetrault (1930). Tetrault's inability to resolve the problem of cellulolytic instability of his culture led him to speculate on the possibility of (1) a life cycle of the organism; (2) change in physiology of the organism; and (3) associated growth of two or more organisms, symbiosis. He also said: "This third theory is the only one which can account for all of the conditions as observed. To accept this symbiotic theory would be an easy way out of a difficult situation. The author is still not convinced that such is actually the case and, at this time prefers to leave the explanation of the phenomenon open."

Enebo's earlier work (1943a, b, 1944, 1948) indicated that cellulose digesters could not grow in pure culture but required the presence of symbionts.

Murray (1944), working with an unnamed thermophilic bacterial culture, believed that humidity enhanced cellulose digestion in agar plates; and that cysteine and other reducing agents seemed to decrease the effectiveness of the cellulolytic activity of the culture in liquid media.
McBee (1950) reported that similar difficulties concerning pure culture isolation, cellulolytic instability, etc. were experienced by virtually every investigator (Kroulik, 1913; Khouvine, 1923; Khouvine and Soeters, 1935a, b; 1936; Langwell and Lymn, 1923; Coolhaas, 1928; Snieszko, 1933; Imsenecki, 1939a, b, 1940a, b; Imsenecki and Bojarskaja, 1939; Rotmistrov, 1939, 1940; Ponchon, 1942a, b) involved in work with anaerobic, cellulolytic, thermophilic bacteria.

Oates et al. (1961, 1963, 1964) described a symbiotic relationship between Bacillus cereus (#33) and Bacillus stearothermophilus (#56). This association, it was reported, produced a two-fold effect: the mesophile, B. cereus was protected by the thermophile, B. stearothermophilus, when grown at elevated temperatures (e.g., 55-65°C); and the symbiotic growth effected cellulose digestion which would not occur when either of the organisms were grown alone.
MATERIALS AND METHODS

Microorganisms

Clostridium thermocellum strain C & E, one of the three bacterial cultures employed in this investigation, was originally isolated, described, and given the name of C. thermocellum by Viljoen, Fred and Peterson (1926). Oates et al. (1961, 1963, 1964) described the culture as a microaerophilic, symbiotic mesophile-thermophile mixture, composed of Bacillus cereus var. mycoides (#33), the mesophilic symbiont and Bacillus stearothermophilus (#56), the thermophilic partner; the strain designations of C & E, #33 and #56, derive from previous studies conducted in this laboratory (Oates, 1964).

Media

Two different media were used for routine cultivation of the bacterial cultures: D58, a basal salts yeast extract medium (Table 1) originally formulated by Quinn (1949); and Jeffers, a modified trypticase soy medium (Table 2). In medium D58, wherever appropriate, either glucose, cellobiose or cellulose was employed as the carbohydrate source at a concentration of \(8.33 \times 10^{-3}\) M for the soluble sugars and 1.5% for cellulose (Microcrystalline cellulose; American Viscose Corp., Marcus Hook, Pa.). The media were sterilized by autoclaving for 15 min at 15# steam pressure and 121C.
Table 1. Composition of D58 medium

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Amounts in g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>((\text{NH}_4)_2\text{SO}_4)</td>
<td>1.3</td>
</tr>
<tr>
<td>(\text{MgCl}_2\cdot6\text{H}_2\text{O})</td>
<td>2.6</td>
</tr>
<tr>
<td>(\text{KH}_2\text{PO}_4)</td>
<td>1.43</td>
</tr>
<tr>
<td>(\text{K}_2\text{HPO}_4\cdot3\text{H}_2\text{O})</td>
<td>7.2</td>
</tr>
<tr>
<td>(\text{CaCl}_2)</td>
<td>0.13</td>
</tr>
<tr>
<td>Yeast extract(^a)</td>
<td>4.5</td>
</tr>
<tr>
<td>Distilled water</td>
<td>qs(^b)</td>
</tr>
</tbody>
</table>

\(^a\)Bacto yeast extract - "Difco".
\(^b\)Quantum sufficit.

Table 2. Composition of Jeffers medium

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Amounts in g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypticase soy agar</td>
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<tr>
<td>Yeast extract(^a)</td>
<td>2.0</td>
</tr>
<tr>
<td>Dextrose</td>
<td>2.0</td>
</tr>
<tr>
<td>Starch (soluble potato)(^b)</td>
<td>2.0</td>
</tr>
<tr>
<td>Agar</td>
<td>7.5</td>
</tr>
<tr>
<td>Distilled water</td>
<td>qs(^c)</td>
</tr>
</tbody>
</table>

\(^a\)Bacto yeast extract - "Difco".
\(^b\)General Chemical Division; Allied Chemical & Dye Corp., New York, N.Y.
\(^c\)Quantum sufficit.
Attempts to Reconstitute Bacillus cereus Var. mycoides (#33) and Bacillus stearothermophilus (#56) into a Mixed Symbiotic Culture

The individual symbionts, cultures #33 and #56, of the C & E mixture were cultivated on agar slants of medium D58 and incubated for 24 hr at 37C and 65C, respectively. The growth on the agar slants was washed off with 5 ml of normal saline (0.85% NaCl). A 0.05 ml volume of each culture was inoculated into each of the triplicate series of 16 x 125 mm screw-capped tubes containing D58-cellulose broth distributed in 6 ml volumes. The tubes were incubated aerobically at 65C for 7-14 days. Behavior of normal controls were indicated by broth growth of culture #56 with the absence of cellulose fermentation; and in the case of culture #33, no growth occurred since, according to reports (Oates et al., 1961, 1963, 1964), the presence of culture #56 was necessary for the growth of this organism at high temperatures.

C & E and #56 culture filtrates

Filtrates from the C & E and #56 cultures were prepared according to the methods of Oates (1964): "C & E culture filtrates which had been grown at 65C for approximately 5 days were centrifuged at 37,000 x g for 45 min in a Servall centrifuge, model SS3, to remove viscous products of the cellulose fermentation. The resulting 'supernatant' was decanted and passed through a millipore filter of 0.3µ porosity to effectively sterilize the cell-free filtrate." Various inoculum
sizes (0.1 ml - 0.5 ml) of saline-resuspended, 24 hr D58 agar slant cultures of #33 were inoculated into duplicate series of 16 x 125 mm screw-capped tubes containing 6 ml volumes of the following media: (1) D58 broth; (2) filter sterilized C & E filtrate; and (3) filter sterilized C & E filtrate combined with D58 broth, at concentrations of 25%, 50% and 75%. The tubes were incubated aerobically at 65°C for 7-14 days. The experiment was performed five times; it was also repeated employing filter sterilized filtrates of the #56 culture.

Combination of high temperature non-cellulolytic C & E isolates

Forty C & E cultures (derived from isolates grown at 65°C) which failed to ferment cellulose were combined in pairs with forty other high temperature, non-cellulolytic C & E isolates in an attempt to obtain a symbiotic, or cellulolytic, culture. In this approach an equal-sized inoculum (.05 ml) of each of a pair of non-cellulolytic cultures were inoculated into 6 ml volumes of D58-cellulose broth contained in 16 x 125 mm screw-capped tubes, and incubated aerobically for 1-4 weeks. The cultures were examined periodically for evidence of cellulose fermentation.

Attempts to Separate the Individual Symbionts from the Mixed Associative Culture of Clostridium thermocellum Strain C & E

A series of forty D58 agar plates were prepared, of which only one-half of the forty plates contained carbohydrate (glucose) as the principal source of energy. Relative to
medium composition, the plates were equally divided in number and incubated aerobically at 37C and 65C for 1-5 days. (Only the plates that were incubated at 65C ever showed any growth, with the emerging colonies being of only one characteristic type, that of the #56 culture.) Numerous colonies were picked and inoculated into 6 ml volumes of D58-cellulose broth distributed in 16 x 125 mm screw-capped tubes, and incubated aerobically at 37C and 65C for 1-4 weeks. The same experiment was performed substituting spore cultures for the vegetative, cellulolytic, C & E cultures.

The foregoing experiments were repeated employing plating media of the following composition: (1) D58 + cellobiose; (2) D58 + cellulose; (3) D58 + various concentrations of filter sterilized broth filtrates taken from active and spent D58-cellulose-grown C & E cultures; and (4) Jeffers medium, with and without glucose.

The Use of Cellulose as a Possible Cellulase Inducer

Preparation of cellulose agar plates

Cellulose agar plates were prepared by the addition of 1.0% partially depolymerized cellulose (Wolf from and Dacons, 1952) to a carbohydrate-free D58 medium containing 1.5% agar. Additional agar plates were prepared employing 1.0% solka-floc BW-200 (Brown Paper Co., Libertyville, Illinois) as the carbohydrate source. Since solka-floc cellulose settled rather rapidly to the bottom of the petri plates, the "agar patties"
were aseptically inverted (via large spatula) and placed in sterile petri plates which contained 1 ml of melted agar for adhesive purposes. The plates were streaked with a cellulose-fermenting C & E broth culture and were incubated aerobically for 24-48 hr. Hundreds of colonies were picked, inoculated into D58-cellulose broth and were incubated aerobically at 65C for 1-2 weeks.

The procedure was repeated substituting Jeffers medium for D58 as the plating medium.

The Response of Clostridium th-amyocellum Strain C & E to Rapid Transfer in Cellulose Broth

The C & E cultures were grown in D58-cellulose broth and were normally carried in 16 x 125 mm screw-capped tubes containing 6 ml of culture medium. An experiment designed to determine the effect of rapid transfer on the cellulolytic activity of the C & E culture was conducted as follows:

A triplicate series of 16 x 125 mm screw-capped tubes, each containing a 6 ml volume of D58-cellulose broth, were inoculated with 0.05 ml of a 48 hr C & E mother culture; these tubes were incubated aerobically at 65C for periods ranging from 6-48 hr. During a 48 hr period, at intervals of 6 hr, culture transfers of 0.05 ml were made into D58-cellulose broths, and the culture tubes were incubated aerobically at 65C. The "daughter cultures" derived from the serial transfers were examined for their ability to ferment cellulose during the course of a 1-2 week incubation period. The experiments were
repeated using D58-cellulose broth containing various concentrations (0.15% - 1.0%) of added filter-sterilized glucose.

Growth of *Clostridium thermocellum* Strain C & E Under Aerobic and Anaerobic Conditions

Serial dilutions of a cellulolytic C & E broth culture were prepared in normal saline. The dilutions were plated in D58 agar which contained cellobiose as the carbohydrate source. The plates were placed in a 19.5" x 7.75" anaerobic jar (Case Laboratories, Inc., Chicago, Illinois), provided with an atmosphere of 5% CO₂ - 95% N₂ and incubated at 65°C for 48-72 hr. The transparent plating medium facilitated rapid detection of different colonial types.

Photography

Photomicrographs were taken of all the types of organisms involved in the present investigation. These organisms were grown in petri plates of cellobiose-containing D58 agar and were incubated under the appropriate growth conditions for 48 hr. The organisms were microphotographed in Gram-stained preparations, and macrophotographed as submerged agar colonies. Photomicrographs of the Gram-stained preparations were taken on 35 mm High Speed Ektachrome (Type B), employing a Leitz microscope, model #250; the submerged agar colonies were taken on 35 mm Ektacolor film (Type L) employing a Leitz microscope (Macro Dia) at a magnification of eight diameters.
Demonstration of Marker Antigens of Component Organisms in Reconstituted Clostridium thermocellum (C & E) by Serological Means

Bacterial cultures

The three bacterial cultures used as antigens were: C. thermocellum strain C & E, a mixed mesophile-thermophile symbiotic culture; B. cereus var. mycoides (#33), the mesophilic symbiont; and B. stearothermophilus (#56), the thermophilic partner.

Growth conditions

The organisms were grown for 48 hr in D58-cellulose broth distributed in 100 ml volumes in 250 ml screw-capped Erlenmyer flasks. Culture #33 was grown at 25°C, while cultures #56 and C & E were grown at 65°C.

Antigens

For the preparation of immunogens, each culture was individually centrifuged, and cells were twice washed by resuspension in isotonic saline followed by centrifugation at 3440 x g for 1/2 hr in a Servall centrifuge, model SP/X. Washed cells were resuspended in saline and diluted to a density matching McFarland Nephelometer tube #2 (Campbell et al., 1964).

Immunization

Three rabbits, each weighing about 3.5 kg, were injected with antigens for a period of time (Table 3) that would normally be expected to insure a substantial level of antibodies. The
Table 3. Animal inoculation schedule

<table>
<thead>
<tr>
<th>Time Day</th>
<th>Injection No.</th>
<th>Volume ml</th>
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<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>0.2</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>0.3</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>0.5</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>1.0</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
<td>1.5</td>
</tr>
<tr>
<td>11</td>
<td>6</td>
<td>2.0</td>
</tr>
<tr>
<td>12</td>
<td>7</td>
<td>2.0</td>
</tr>
<tr>
<td>15</td>
<td>8</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Antigens were injected via dorsal marginal ear veins of the rabbits with 27 gauge needles attached to 1 ml tuberculin syringes.

Five days following the final injection, 20 ml of blood was obtained from each rabbit via cardiac puncture. This was accomplished with the use of an 18 gauge needle attached to a 20 ml syringe. The whole blood was allowed to coagulate at room temperature and was then separated by mild centrifugation to remove the sera from the cell debris.

Immunodiffusion

The soluble antigens used for immunodiffusion were prepared as follows:
Cultures #33, #56 and C & E were grown separately in 250 ml screw-capped Erlenmyer flasks containing 100 ml volumes of D58-cellulose broth. The cultures were incubated at their optimal temperatures for 72 hr: culture #33 at 25°C and cultures #56 and C & E at 65°C. Following incubation the cultures were differentially centrifuged, first at 34 x g for 1/2 hr to remove the cellulose, then at 13,800 x g for 1/2 hr to remove the cells. The supernatant, which contained cell-free autolysate, was retained and served as the soluble antigen.

The preparation of Ouchterlony gel diffusion plates was carried out as described by Campbell et al. (1964). Each of three plates was arranged with one of the antigens in the center well, with duplicates of the three antisera in the diametrically paired peripheral wells. The center wells contained 0.1 ml antisera. The plates were incubated at 37°C in a moist chamber for 3-4 days before precipitin lines developed.

Photography

The photographs of the Ouchterlony plates were prepared by Photo Services, B-12 Morrill Hall, Iowa State University, Ames, Iowa.
RESULTS AND DISCUSSION

Demonstration of the Absence of a Symbiotic Relationship between "Bacillus stearothermophilus" and Bacillus cereus

Fundamental to any research project are the preliminary experiments which permit one to become acquainted with the idiosyncrasies of the problem. With this in mind, the study of the "thermophily factor" was pursued by first attempting to reconstitute the individual symbionts into their associative, cellulolytic, mixture. The two organisms, Bacillus cereus (#33) and Bacillus stearothermophilus, were inoculated into D58-cel-lulose broth in approximately equal proportions, incubated aerobically at 65°C for 7-14 days and examined periodically for evidence of cellulose fermentation. Excellent growth appeared in the culture tubes containing the newly inoculated mixture, but with no evidence of cellulose fermentation. The control tubes which contained only culture #56 showed good growth, while those control tubes with only culture #33, as would be expected, showed no growth. The positive (or cellulolytic) con-trol, which contained the C & E culture, showed fermentation after 24-48 hr, as indicated by vigorous gas evolution with concomitant chromogenesis of the cellulose.

At this point it appeared that a plausible explanation for inability to obtain a reconstituted cellulolytic mixture might be attributed to failure of the mesophile (#33) to grow at 65°C. Therefore to explore the possibility that the mesophile was not
17

growing at these elevated temperatures, experiments were conducted which employed filter sterilized broth filtrates from the #56 and C & E cultures. Culture #33 was inoculated into freshly prepared D58-broth filtrates and incubated at 65C; however, every effort to demonstrate growth of the mesophile at high temperatures, employing this technique, were unsuccessful.

Another, seemingly remote, possibility that might have served as an explanation for the inability to combine cultures #33 and #56 into a cellulolytic mixture was considered: that of an incompatibility between the two cultures having occurred, and perhaps attributable to a long term separation of the two organism from one another. It seemed, therefore, that if that were the case the problem might be circumvented by obtaining new isolates from the C & E culture. But every attempt to effect a separation of the two organisms from the C & E culture, employing the techniques described in the Methods section, were again unsuccessful. Growth could be obtained only from the agar plates which had been incubated at high temperatures.

An interesting observation was made, however. Although plates streaked with the C & E culture gave rise to colonies only when they were incubated at high temperatures (e.g. 55C-65C), some of the primary isolates were found to be capable of fermenting cellulose when returned to cellulose broth; but if those same primary, cellulolytic, colonies were streaked onto secondary plates of agar, prior to being returned to D58-cellulose broth, the resulting "daughter colonies" permanently
lost their ability to ferment cellulose. These were the same observations that were made by virtually every pioneer investigator of cellulose-digesting bacteria, and which eventually led some workers to believe that growth of the organism in the presence of cellulose was necessary for the maintenance of cellulolytic activity.

In an attempt to explain the difficulties which were being encountered with *C. thermocellum* (C & E) three hypotheses were proposed:

1. The cellulolytic isolates were composed of two organisms, a mesophile and a thermophile, with the mesophile being capable of growth at high temperatures only when in close proximity to the thermophile;  
2. the cellulolytic isolates were composed of two aerobic thermophiles which, when separated from one another, resulted in a culture which was unable to ferment cellulose;  
3. The C & E culture consisted of only one type of organism, and its cellulolytic stability was dependent upon continued cultivation in the presence of cellulose.

The first of the three hypotheses was not overly appealing since previous attempts to separate the mesophile from the C & E mixture were fruitless. If the second hypothesis were correct it ought to be amenable to simple demonstration. Therefore in an attempt to demonstrate hypothesis #2, forty non-cellulolytic isolates were combined in pairs in D58-cellulose broth with forty other non-cellulolytic thermophiles. It was hoped that at least one mixed culture-combination would be capable of
fermenting cellulose. The result of such an experiment was that good growth occurred in all of the culture tubes, but without fermentation of cellulose.

Cellulose as a Possible Cellulase Inducer

The third hypothesis (the C & E culture consisted of only one type of organism, and its cellulolytic stability was dependent upon continued cultivation in the presence of cellulose) was prompted by a report in the literature which considered the presence of cellulose in the medium essential for the maintenance of cellulolytic activity of cellulose digesting bacteria (Viljoen et al., 1926); and as a consequence of that report an experiment to test hypothesis #3 was designed. Cellulose agar plates were prepared in such a way as to obtain isolates that had not lost direct contact with cellulose during growth. This approach did not, however, alleviate the problem of cellulolytic instability.

Up to this point every effort to arrive at a reasonable explanation for the unconventional behavior of C. thermocellum (C & E) had failed. More distressing, however, was the fact that most of the observations made by this investigator were no more advanced than those which were made by workers of forty years ago. This awareness, combined with almost two years of what appeared to be complete failure, would have caused any prudent graduate student to immediately abandon this problem. Notwithstanding, the investigation was continued, but prompted
only by a combined feeling of humility and challenge.

Conditions Which Influence the Cellulolytic Activity of *Clostridium thermocellum* (C & E)

The culture medium normally employed for the routine cultivation of the C & E culture was D58 broth supplemented with cellulose as the carbohydrate source. When fresh working cultures were prepared, they were usually allowed to incubate for about 48 hr, or until fermentation appeared to be well underway, as evidenced by vigorous gas evolution in the culture broth. It was noted that accompanying the onset of fermentation (24-48 hr) was the appearance on the surface of the cellulose of a golden to canary yellow chromogenesis which became progressively intense in color during the course of fermentation (Figure 1-D). The overlying broth usually became turbid at about 3-6 hr following incubation.

When frequent transfers of the C & E cellulose broth culture were made, as for example at 6 hr intervals, the broth cultures derived from the early transfers grew excellently but irretrievably lost their ability to ferment cellulose. Broth cultures which were transferred beyond 24 hr of incubation were almost invariably cellulolytic cultures. Noteworthy was the observation that if, just prior to transfer, the broth cultures were vigorously shaken to cause a disturbance of the entire contents of the culture tube, the frequency of the cellulolytic cultures obtained in the serial transfer experiment was enhanced.
Figure 1. D58-cellulose broth cultures depicting the following: A: Uninoculated control; B: C. thermocellum at 24 hr; C: C. thermocellum at 48 hr; D: C. thermocellum strain C & E (mixed culture) at 48 hr; E: "B. pumilus" at 48 hr
If the cellulose broth contained an added amount of glucose (e.g., 0.15%-1.0%), as was done on several occasions, earlier growth was observed but fermentation and chromogenesis appeared to be suppressed relative to the increase concentration of glucose added to the growth medium. Moreover, when serial transfers of these glucose-containing broth cultures were made between the 18th to the 24th hr of incubation, the resulting "daughter cultures" were more frequently unable to ferment cellulose than the ones derived from the cultures lacking the added glucose.

Growth of Clostridium thermocellum (C & E) Under Aerobic and Anaerobic Conditions

Procedures involving strict anaerobic growth conditions were adopted after having read several reports (McBee, 1948, 1950) in which C. thermocellum was considered to be an obligate anaerobe and of one species.

The C & E culture was serially diluted in normal saline, plated in cellubiose-containing D58 agar and incubated anaerobically at 65°C. After 48 hr of incubation two distinct, submerged, colonial types developed: (1) white to golden lenticular colonies (Figures 2 & 3); and (2) white to orange colored, "feathery", types (Figure 3). Additional colonial types did not develop with prolonged incubation of the agar plates. The two different colonial types were picked, reinoculated into cellubiose-containing D58 agar and incubated at 65°C under both
Figure 2. Photomicrograph showing a broad view of two submerged agar colonies from a pure culture of *Clostridium thermocellum*. X8
Figure 3. Photomicrograph showing submerged agar colonies of two associative bacteria which constitute the culture of Clostridium thermocellum strain C & E. The lenticular colony at left is C. thermocellum; the "feathery" colony at right is "Bacillus pumilus" X8
aerobic and anaerobic conditions. The isolation procedure was repeated four times to ascertain purity of the two colonial types.

It was noted that the lenticular colonies developed only when they were grown under anaerobic conditions, whereas the "feathery" colonies developed under both aerobic and anaerobic conditions. Following the purification procedure, the two isolates were returned to D58-cellulose broth and incubated at 65°C under aerobic and anaerobic conditions. During a 48-72 hr incubation period the pure cultures were examined for their ability to grow and ferment cellulose. The white to golden lenticular colony type proved to be an obligate anaerobe, which grew and fermented cellulose under anaerobic conditions either in pure culture or when mixed with culture #56. The "feathery" colony isolate was identified as culture #56 and turned out to be a facultative anaerobe. When the two cultures were grown separately (in D58-cellulose broth) under aerobic conditions, only culture #56 grew. When the two cultures were recombined, growth and fermentation of cellulose occurred either under aerobic or anaerobic conditions.

Identification, Description and Classification of the Cellulolytic Mixture of Clostridium thermocellum Strain C & E

Biochemical tests were performed on the cellulolytic, obligate, anaerobe and it was identified as Clostridium thermocellum (Bergey's 7th ed.), as had previously been described for the
pure culture (McBee, 1950, 1954). See Figures 4, 5, and 6 for photomicrographs taken of Gram-stained preparations of a pure culture of *C. thermocellum*.

This laboratory's culture of *C. thermocellum* came from the laboratory of Viljoen (University of Wisconsin) via Dr. P. A. Tetrault (Purdue University), from whom McBee also obtained his culture (McBee, 1950). However all of McBee's studies employed strict anaerobic conditions; and upon receiving his culture from Tetrault, he cultivated the organism under anaerobic conditions in transparent, cellobiose-containing, agar and was able to immediately recognize and select only the cellulose fermenters while eliminating any of the non-cellulolytic, associative forms.

The *B. cereus* var. *mycoides* and "*B. stearothermophilus*" cultures were sent to Dr. Ruth Gordon (Rutgers University) for identification. The *B. stearothermophilus* culture was identified as a typical strain of *Bacillus pumilus*, an organism which is commonly associated with plant material (Figures 7 & 8). Dr. Gordon failed to mention anything about temperature relationships in her identification of our organism. *B. pumilus* is described as having a temperature relation of 28-40C as optimum and 45-50C as maximum (Bergey's 7th ed., 1957); however, our organism appears to be an obligate thermophile and grows luxuriantly at 65C. Deriving from information obtained in a previous investigation (Oates, 1964) there is evidence that this organism (#56) may be a new species of the genus *Bacillus*. 
Figure 4. Gram-stained preparation of *C. thermocellum* showing elongated cells with terminal spores. x 1000
Figure 5. Gram-stained preparation of *C. thermocellum* as seen via dark field illumination. x 1000
Figure 6. Gram-stained preparation of *C. thermocellum* as seen via dark field illumination. Note both sporangial and vegetative cells. x 1000
Figure 7. Gram-stained preparation of "B. pumilus" showing a typical scattered cellular arrangement. x 540
Figure 8. Gram-stained preparation of B. pumilus as seen via dark field illumination. x 1000
The taxonomic issue, however, was not pursued any further.

The culture of \textit{B. cereus} var. \textit{mycoides} was identified as a typical amycoidal strain of \textit{B. cereus} (Figures 9, 10 & 11).

\textbf{Serology}

The absence of the mesophile (#33) from the C & E culture was further substantiated by an investigation which employed serological methods (Quinn and Pemberton)\textsuperscript{1}. As shown in Figure 12, the center well contains #33 antigen, with its homologous antiserum in the two opposite peripheral wells indicated by the number 1. The wells indicated by the numbers 2 and 3 contain #56 and the C & E antigens, respectively. It will be noted that the homologous system shows two sharp bands with no cross reactions between #56 and C & E antisera. These results would be expected if the #33 culture (or mesophile) were absent from the C & E culture.

The immunodiffusion plate in Figure 13 contains the #56 antigen in the center well with the homologous antiserum in the two opposite peripheral wells, indicated by the number 2. The wells with the numbers 1 and 3 contain the antisera of #33 and C & E, respectively. In this plate there is a strong reaction between the homologous system which shows three distinct precipitin bands; two of those bands show reactions of identity with bands formed between C & E antiserum and #56 antigen. No

\textsuperscript{1}Quinn and Pemberton (unpublished results), Department of Bacteriology, Iowa State University, Ames, Iowa.
Figure 9. Gram-stained preparation of *B. cereus*. Note the characteristic chain-like cellular arrangement. x 540
Figure 10. Gram-stained preparation of B. cereus (#33) as seen via dark field illumination. x 1000
Figure 11. Photomicrograph showing a submerged agar colony of *Bacillus cereus* (#33). x 8
Figure 12. An Ouchterlony gel diffusion plate showing antigen #33 in center well with homologous antiserum in the two peripheral wells, as indicated by the number 1. Well numbers 2 and 3 contain antisera #56 and C & E, respectively.
Figure 13. An Ouchterlony gel diffusion plate showing antigen #56 in center well with homologous antiserum in the two peripheral wells, as indicated by the number 2. Well numbers 1 and 3 contain antiserum #33 and C & E, respectively.
reaction is observed between #56 antiserum and #33 antigen.

The immunodiffusion plate in Figure 14 contains C & E antigen in the center well and homologous antiserum in the two opposite wells, as indicated by the number 3. The wells designated by the numbers 1 and 2 contain the antisera of #33 and #56, respectively. It will be noted that the C & E antigen reacts to form numerous bands with the homologous antiserum, showing some lines of identity formed with the #56 antiserum. No reaction is seen between #33 antigen and C & E antiserum.

The foregoing serological data is further evidence that the mesophile, or #33, is absent from the reconstituted C & E culture; and, that culture #56 ("B. pumilus") is an integral part of the associative mixture comprising C. thermocellum strain C & E.

I should now like to dwell upon some of the difficulties which were encountered in the earlier part of this investigation by first commenting on the mesophile, or #33, culture. This organism was apparently isolated as a contaminant of the C. thermocellum (C & E) stock cultures, and was later believed to be a functional part of the cellulolytic, associative mixture, which is now known to consist of C. thermocellum and "B. pumilus". Attempts to grow the mesophile at elevated temperatures were unsuccessful, and its presence was not essential to effect the fermentation of cellulose (at 55C-65C) either under aerobic or anaerobic conditions when a mixture of the other two
Figure 14. An Ouchterlony gel diffusion plate showing C & E antigen in center well with homologous antiserum in the two peripheral wells, as indicated by the number 3. Well numbers 1 and 2 contain antisera #33 and #56, respectively.
organisms, #56 and *C. thermocellum* (the obligate anaerobe) were employed.

One may recall from earlier experiments that some of the primary agar plate colonies from C & E cultures were sometimes cellulolytic; but if those same cellulolytic colonies were restreaked onto secondary agar plates, the emerging colonies gave rise to cultures which had irretrievably lost their ability to ferment cellulose. It is now obvious that the reason for cellulolytic cultures arising from the primary agar plate isolates was due to a mixture of *"B. pumilus"* and *C. thermocellum*, since only a combination of these two organisms ever gave rise to cultures which were capable of fermenting cellulose under aerobic growth conditions.

Of interest was the observation that an occasional C & E spore culture could be found which consistently gave rise to cellulolytic colonies (when taken from primary agar plates) at the rate of > 95%. Apparently the isolates were mixed with *C. thermocellum* and *"B. pumilus"*. The question was: Why should there have been such a consistently high frequency of mixed colonies? Is it possible that the spores of the mixed culture have an affinity for each other (perhaps mediated by charge) which permits the organisms to retain a "symbiotic relationship" even in the absence of active growth? This may be a plausible explanation if one considers that the two organisms do have an interesting relationship; and that strict anaerobiasis is not readily found in nature. It was also frequently noted that the
better developed agar colonies of *C. thermocellum* were the ones which were in close proximity to the *Bacillus* species.

The observations made that the C & E broth cultures irretrievably lost their cellulolytic activity upon being frequently transferred during early periods of growth, are no longer enigmatic. It has been observed (deriving from information in the second section of this dissertation, and other sources [McBee, 1948, 1950]) that when a pure culture of *C. thermocellum* is grown in cellulose broth the organisms become attached to the cellulose fibers (by some unknown means) and commence to multiply, while the overlying broth remains relatively clear until the advanced stages of cellulose fermentation. Furthermore, the lag phase period for this organism is considerably longer than that observed for the *Bacillus* species. In retrospect, then, the reason for having obtained non-cellulolytic cultures during the rapid transfer experiments is explainable on the basis of earlier growth of the *Bacillus* species. Moreover, the addition of glucose to the cellulose broths, while enhancing earlier growth of "*B. pumilus*", caused a substantial reduction in pH (pH 6.9 to 5.45) resulting in a suppressive effect to the growth of the obligate anaerobe, *C. thermocellum*. It has long been known that pure cultures of anaerobic, cellulolytic thermophiles will not grow in medium having a pH value of less than 6.5 (McBee, 1948, 1950; Enebo, 1954).
The increased frequency of cellulolytic cultures (in the rapid transfer experiments) resulting from the vigorous shaking of the C & E mother cultures just prior to transfer, is attributed to the presence of cellulose particles in the inoculum to which cells of *C. thermocellum* were attached.

From the evidence presented in this investigation there can be little doubt concerning the true nature of the associative mixture of *C. thermocellum* (C & E).

It is probable that the difficulties which plagued pioneer investigators in their pursuit of pure or stable cellulolytic cultures was the result of, as in the present investigation, more vigorous activity of non-cellulolytic, associative, bacteria which eventually predominated in their cultures, resulting in the isolation of organisms which lacked the ability to ferment cellulose.
SUMMARY

It has been reported (Oates et al., 1961, 1963, 1964) that Clostridium thermocellum strain C & E is composed of a mixed mesophile-thermophile culture which symbiotically digests cellulose at 65°C. The mesophile was identified as Bacillus cereus var. mycoides (#33) and the thermophile as Bacillus stearothermophilus (#56).

This investigation has demonstrated that the C & E culture is, instead, composed of two thermophiles: a non-cellulolytic, facultative anaerobe which has been identified as "Bacillus pumilus"; and a cellulose-digesting, obligate anaerobe identified as Clostridium thermocellum. In association, the two organisms digest cellulose either aerobically or anaerobically; separately, the Clostridium requires strict anaerobic conditions for growth and fermentation of cellulose.

The presence of B. cereus var. mycoides (#33) as an integral part of the C & E culture was investigated, as well as the "thermophily factor" which was responsible for its growth at high temperatures. The B. cereus var. mycoides was identified as an amyloid strain of B. cereus, and was found to have no relation to the C & E culture. Attempts to demonstrate a "thermophily factor" in filtrates of cultures #56 and C & E were unsuccessful.
PART II. THE UTILIZATION OF HEXOSES BY

CLOSTRIDIUM THERMOCELLUM
INTRODUCTION

The preference for cellobiose over glucose is not uncommon among cellulolytic bacteria. Yet despite the preference for the disaccharide sugar most of these organisms ferment glucose, or possess the necessary enzyme-systems for such.

A number of reports involving studies with Clostridium thermocellum have shown that although this species was capable of fermenting cellulose and cellobiose, it failed to utilize glucose or any of the related hexoses; however, glucose was always found in spent, cellulose-grown, cultures when the concentration of cellulose exceeded 0.15 percent. This behavior, appearing anomalous with regard to glucose (when one considers the nature of cellulose as anhydroglucose), prompted McBee (1948, 1950, 1954) and others (Sih and McBee, 1955; Sih et al., 1957; Nelson and McBee, 1957) to devote a considerable amount of time exploring this phenomenon. However, in spite of their efforts, the phenomenon remained unresolved.

This seemingly anomalous behavior of C. thermocellum toward glucose attracted our attention, and a project directed at explaining this phenomenon was initiated. Part II of this thesis is, therefore, devoted to physiological peculiarities of three strains of C. thermocellum (strains TET, 157 and 651), all of which were previously studied by R. H. McBee (1950); also, taxonomic considerations are proposed for C. thermocellum, relative to the information obtained in this study.
R. H. McBee (1948, 1950), employing the techniques of R. E. Hungate (1944, 1947), was able to successfully and unequivocally demonstrate that cellulose-digesting thermophilic bacteria were amenable to isolation in pure culture; and that the capacity to ferment cellulose was, in fact, retained even when the organisms were cultivated in the absence of cellulose, a subject that had been controversial in earlier years. The main advantage of their techniques over those used by earlier investigators was the employment of cellobiose as the carbohydrate source, together with more stringent anaerobic growth conditions. The use of cellobiose as the principal energy source permitted the preparation of transparent plating medium and thus facilitated the isolation of pure colonies.

McBee obtained all available cultures of anaerobic, thermophilic, cellulolytic bacteria and proceeded to study them in considerable detail. He found that the cultures showed such marked similarities in morphology and physiology that he suggested the possibility of including all of the known anaerobic, thermophilic, cellulose-fermenting bacteria into one group, namely, Clostridium thermocellum (1950, 1954). A peculiar behavior of these organisms was observed: although they were capable of fermenting both cellulose and cellobiose, they failed to utilize glucose or any of the related hexoses. Attempts to explain this anomalous behavior toward glucose was vigorously
pursued then and in subsequent years.

Considering the possibility that reagent-grade glucose might possess subtle chemical differences from that found in digested cellulose material, McBee (1948) isolated, purified and employed glucose from spent, cellulose-digested, broth cultures. The results were the same: a continued absence of growth in glucose-containing medium. He considered two other possibilities to account for this behavior: (1) that glucose fermentation occurred only when the organisms were growing in the presence of cellulose or cellobiose; and (2) that the glucose in the spent cultures was the result of acid hydrolysis in the latter stages of cellulose fermentation. The results of his experiments, however, indicated that this was not the case.

Additional work (Sih and McBee, 1955; Alexander, 1961) on the physiology of C. thermocellum led to the isolation and characterization of cellobiose phosphorylase, an intracellular enzyme which cleaves cellobiose to yield glucose and glucose-1-phosphate. This enzyme also works in reverse and has been shown to catalyze an in vitro reaction between D-glucose and α-D-glucose-1-phosphate leading to the formation of cellobiose (Sih and McBee, 1955; Alexander, 1968a). Also identified, but not isolated, was a glucokinase which, in the presence of ATP, converts glucose to glucose-6-phosphate (Sih and McBee, 1955; Alexander, 1968a). The presence of glucokinase in this organism, together with the observation that glucose could not be found
in broth filtrates from cultures grown on small amounts (0.15%) of cellulose, prompted the following hypothesis by Sih and McBee (1955): the kinase in the living system works simultaneously with the phosphorylase to form two glucose-phosphates; or, that there is also a cellobiokinase that forms a cellobiose phosphate which is then split by cellobiose phosphorylase (via phosphorolysis) to give two glucose phosphates.

The presence of a cellobiokinase was later demonstrated by Nelson and McBee (1957) but the location of the phosphate on the cellobiose phosphate molecule was not determined. The finding of a cellobiokinase obviously strengthened the hypothesis by Sih and McBee (1955) to account for the absence of glucose in filtrates derived from cultures grown on small amounts of cellolose. Nonetheless, the question of why C. thermocellum failed to ferment glucose remained unresolved.

The preference of cellulose and cellobiose over glucose is peculiar to cellulolytic bacteria (Hutchinson and Clayton, 1918; Enebo, 1949; Hungate, 1950; Sjöpristeijn, 1951; Hulcher and King, 1958). It has been reported that most strains of Ruminococcus flavefaciens fail to ferment glucose, despite the evidence that this organism possesses a glucokinase (Ayers, 1958).

Hulcher and King (1958) have described an anaerobic, mesophilic, cellulolytic bacterium, Cellvibrio gilvus n. sp., which, although capable of fermenting glucose, shows a preference for cellobiose as indicated by the relatively greater overall cell
density and the shorter time required for onset of growth with cellobiose over glucose as the energy source. Their results indicated that the basis for a difference in growth response was due to intracellular metabolism rather than to permeability. With the demonstration of a cellobiose phosphorylase the metabolic basis for this preference was explained by the hypothesis that while glucose was directly oxidized to gluconic acid, cellobiose was cleaved phosphorolytically and further metabolized by reactions leading to a more complete oxidation and therefore to a greater yield of energy per mole of hexose consumed. The hypothesis of a metabolic nonequivalence of the two glucose moieties of cellobiose was later tested and found to be correct (Swisher et al., 1964). In view of the preference shown for cellobiose by C. gilvus n. sp., it is of interest that this organism degrades cellulose by removal of terminal units, yielding only cellobiose as a reaction product (Hulcher and King, 1958).

Enebo, in 1954, working with Clostridium thermocellulaseum, an anaerobic, cellulolytic thermophile, which shares virtually all of the characteristics of C. thermocellum, demonstrated that it, too, preferred cellobiose to monosaccharides. Both glucose and fructose were fermented, but at a much slower rate than cellobiose. He speculated that this effect might be related to the chemical nature of cellulose in which cellobiose as well as glucose may be considered to constitute the ultimate unit.
The role of glutamine in glucose metabolism was studied with a mutant strain of *Clostridium tetani* (Lerner and Mueller, 1949). It was noted that cells which had been grown in iron-rich medium fermented glucose, whereas cells harvested from iron-deficient medium lacked this ability. Glutamine was able to induce glycolytic activity only in iron-deficient cells, as measured by CO₂ evolution. The degree of stimulation was proportional to increased levels of glutamine added. Moreover, excess glutamine increased the Qco₂ almost to the level of high-iron-grown cells. Cell-free extracts from cells grown in media containing high levels of iron yielded a stimulatory substance which was free from protein and apparently of small molecular weight. Further studies showed that the factor stimulating glucose-fermenting activity could be extracted from yeast, mammalian liver, trypticase digest of casein and muscle. It was suggested that the stimulatory activity noted in extracts of high-iron-cells, yeast, liver, etc., was due to the presence of glutamine, either free or in peptide linkage.

Glutamine has also been reported (McIlwain, 1946) to have a stimulatory effect on the glycolytic activity of several strains of hemolytic streptococci. The addition of this compound was found to stimulate the production of lactic acid from glucose by washed cell suspensions.

Field and Lichstein (1958b) reported the influence of casein hydrolysate and amino acids on glucose fermentation by
washed cells of Propionibacterium freudenreichii. Of the amino acids tested only aspartate and asparagine were stimulatory. It was speculated that the stimulatory effect might be explained on the basis of increased permeability of glucose to the cell in the presence of these amino acids.

The first clear instance of the formation of specific enzymes in response to the presence of specific substrate came from the work of Stephenson and Strickland (1932, 1933) with formic hydrogenlyase. They noted that when washed Escherichia coli cells (grown in the absence of formate) were suspended in formate, the enzyme, hydrogenlyase, was produced within 2 hr -- the only requirement being the presence of broth medium, since adaptation occurred poorly in formate medium alone. Moreover, the phenomenon occurred in the absence of cell multiplication.

Evans et al. (1941) reported that tryptophanase, the enzyme responsible for the conversion of tryptophane to indole, is similarly induced when E. coli, grown without tryptophan, is suspended in a solution of this amino acid; no other substrate was required, and no growth in terms of increase of cell numbers occurred.

The term "permease" was first suggested by Cohen and Monod (1957) to denote (in bacteria) stereospecific permeation systems, functionally specialized and distinct from metabolic enzymes. A number of investigations (Monod et al., 1951; Hogness et al., 1955; Monod, 1956) employing radioactive compounds
indicated that induction of a specific transport system for β-galactosides could be effected in *E. coli* if the organism was grown in the presence of a suitable inducer, such as lactose. Non-induced cells (i.e., cells grown in the absence of a galactoside) failed to show any significant levels of radioactive substrate-accumulation.

That the inducible enzyme system of β-galactosidase required active protein synthesis was demonstrated by Munier and Cohen (1956). These workers showed that induction is effected only under conditions which allow for the synthesis of protein, and is blocked by inhibitors of protein synthesis such as chloromycetin, or in the absence of a required amino acid. More importantly, the inducible system was blocked when exposed to β-2-thienylalanine, a compound whose incorporation does not inhibit protein synthesis in *E. coli*, yet renders the newly synthesized protein inactive.

However, the most unequivocal evidence attesting to permeases being distinct and specific functional entities, emerged from the genetic studies of Jacob and Monod (1961). Their studies with *E. coli*, employing lactose as a substrate, showed that the induction process for the β-galactoside permease was dependent upon the presence of a "y" gene in the lactose operon, which governed its formation and thus permitted β-galactosides to enter the cell.
More recent investigations have provided additional information on the subject of permeases, or membrane transport proteins. Pardee et al. (1966, 1967) have isolated and crystallized a sulfate binding protein from *Salmonella typhimurium*. A protein from *E. coli* which binds neutral amino acids also has been isolated and crystallized by the group in Oxender's laboratory (Piperno, and Oxender, 1966; Penrose et al., 1968). Proteins that bind calcium have been isolated from several tissues of chicks and rats (Wasserman and Taylor, 1966; Taylor and Wasserman, 1967; Wasserman et al., 1968).

The area of study concerned with membrane transport proteins is relatively new, but recent literature on this subject is rather extensive. For a brief, but excellent, review on this subject, the recent publication by Pardee (1968) is recommended.
MATERIALS AND METHODS

Microorganisms

The three strains of *Clostridium thermocellum* employed in this investigation were *C. thermocellum* (157), courtesy of Dr. R. H. McBee, Montana State University; *C. thermocellum* (TET), a strain originally obtained from Dr. P. A. Tetrault, Purdue University; and *C. thermocellum* (651), courtesy of Dr. J. K. Alexander, Hahnemann Medical College, Philadelphia, Pennsylvania. The three strains of this organism behaved similarly; however, most of the quantitative work was done with strain 157, since most of the literature on *C. thermocellum* involved this strain.

Media and Growth Conditions

Two basal salts media were employed in these studies: medium D58 (Table 1) originally developed (Quinn, 1949), and subsequently modified, by Quinn (1955); and a basal salts yeast extract (Y.E.) medium originally formulated by R. E. Hungate (Table 4) and later adopted by R. H. McBee for the cultivation of cellulolytic thermophiles. For our purpose, Hungate's medium was subjected to filter sterilization (Millipore; .45 micron porosity) instead of autoclaving. The term "Hungate's modified medium (HMM) or Hungate's modified formula," will be used to indicate that a yeast extract concentration of 0.45% has been substituted for the original 0.05%, as employed by McBee (1948, 1950). Medium D58 was routinely sterilized for 15 min, at 15°
Table 4. Composition of Hungate's minimal medium

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Amounts in g/L</th>
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<tbody>
<tr>
<td>NaCl</td>
<td>6.0</td>
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<tr>
<td>CaCl₂</td>
<td>0.1</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>0.1</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.5</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.5</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>1.0</td>
</tr>
<tr>
<td>Yeast extract&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.5</td>
</tr>
<tr>
<td>Sodium thioglycollate</td>
<td>0.45</td>
</tr>
<tr>
<td>Distilled water</td>
<td>qs&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Bacto yeast extract - "Difco".

<sup>b</sup>Quantum sufficit.

steam pressure and 121°C. The final concentration of carbohydrate used for tests with soluble sugars was \(8.33 \times 10^{-3}\) M, and 1.5% for cellulose (Microcrystalline cellulose; American Viscose Corp., Marcus Hook, Pa.). Whenever sodium thioglycollate (reducing agent) was employed in medium D58, it was added aseptically to autoclaved medium to give a final concentration of 0.04%; when it was employed with HMM, it was added prior to filter-sterilization to give a final concentration of 0.04%.

All experiments were conducted at 60°C under anaerobic conditions.
in a 19.5" x 7.75" anaerobic jar (Case Laboratories, Inc., Chicago, Illinois), provided with an atmosphere of 5% CO₂ + 95% N₂.

Conditions in Medium D58 Which Influence the Growth and Utilization of Hexoses by Clostridium thermocellum

Procedure for Zymosan test

An experiment employing Zymosan, a commercial preparation (Standard Brands, Incorp., 625 Madison Ave., N.Y., N.Y.) of insoluble yeast cell wall material, was conducted as follows:

Experiment no. 1 Zymosan, to give a final concentration of 1 mg per ml was added to a triplicate series of 16 x 125 mm screw-capped tubes, each containing 6 ml of medium D58. The culture tubes were autoclaved for 15 min at 15# steam pressure and 121C. Filter-sterilized glucose was added aseptically to each tube. These tubes were inoculated (0.05 ml) with a 48 hr culture of C. thermocellum (TET) growing in celllobiose-containing D58 medium. Controls consisted of the following; D58 without added glucose; D58 medium + glucose (without Zymosan); D58 + Zymosan (without added glucose); and D58 + celllobiose. All culture tubes were incubated for 1 week.

Experiment no. 2 The previous experiment with Zymosan was repeated, substituting fructose as the carbohydrate source.

Experiment no. 3: heated glucose D58 broth containing glucose as the principal carbohydrate source was distributed in 6 ml volumes in a triplicate series of 16 x 125 mm screw-capped
tubes. The broth tubes were autoclaved for 15 min at 15# steam pressure and 121C. These culture tubes were then inoculated with 0.05 ml of an actively growing mother culture of *C. thermocellum* grown in HMM + cellobiose. Controls consisted of D58 medium without added carbohydrate; D58 medium + filter-sterilized glucose; and D58 medium + filter-sterilized cellobiose. All culture tubes were incubated for a period of 1 week.

**Experiment no. 4** The foregoing experiment was repeated using slightly modified conditions: the D58 broth was autoclaved without added carbohydrate, followed by the addition of glucose that had been autoclaved in distilled water for 15 min at 15# steam pressure and 121C.

**Experiment no. 5: reducing agent** D58 broth was prepared and distributed in 6 ml volumes into each of sixteen 16 x 125 mm screw-capped tubes. Filter-sterilized sodium thioglycollate was added aseptically to all but four of the culture tubes (which served as non-reduced controls). These cultures were arranged in four series of four tubes each, with one tube in each series containing no added reducing agent. The first, second, and third series of culture tubes received filter sterilized glucose, fructose and cellobiose, respectively; the fourth series of four tubes received no added carbohydrate and served as carbohydrate-free controls. All culture tubes were then inoculated with 0.05 ml of an actively growing, HMM-cellobiose grown, mother culture and were incubated for 1 week.
Carbohydrate Determinations

Test for glucose

Glucose was determined by means of the Glucose Oxidase Test (Washko and Rice, 1961). This specific method employs the simultaneous activity of two enzymes, glucose oxidase and peroxidase. The glucose oxidase catalyzes the oxidation of glucose, by molecular oxygen, to gluconic acid and hydrogen peroxide. The hydrogen peroxide formed in the presence of peroxidase and a chromogenic hydrogen donor, o-dianisidine, forms a colored substance which, in an acidic medium, is stoichiometrically related to the amount of glucose in the sample.

Reagents

Barium hydroxide, 0.3N

Into a liter flask place 600 ml of water and boil for 5 min. Add 45 gm of barium hydroxide, Ba(OH)$_2$•$8\text{H}_2\text{O}$, reagent grade, and continue boiling until it is dissolved. Filter the solution rapidly to remove barium carbonate, cool and dilute to a liter. Store in a Pyrex bottle and protect from the air.

Zinc sulfate, 5.0% (w/v)

Dissolve 50.0 gm of zinc sulfate ZnSO$_4$•7H$_2$O, reagent grade, in water and dilute to 1 liter. The accuracy of the concentrations of solutions 1 and 2 is less important than the requirement that they must neutralize each other, volume for volume, when titration is performed with phenophthalein as an indicator. Pipett 10.0 ml of zinc sulfate
solution into a flask and add 100 ml of water. Add a few drops of phenolphthalein indicator (1% in ethyl alcohol). Titrate with the barium hydroxide solution dropwise, with constant agitation until the solution turns pink and the color persists for about one min. On the basis of this titration, the solution that is more concentrated is diluted to match the other.

**Buffered glycerol** To 600 ml of 0.04 M phosphate buffer, pH 7.0, (3.48 gm Na₂HPO₄ and 2.12 gm KH₂PO₄ per liter) add 400 ml of glycerol and mix. This solvent may be kept in a refrigerator for more than a month without any evidence of bacterial contamination.

**Commercial "Glucostat" reagent** Dissolve the contents of the smaller vial in 1.0 ml of water. Dissolve the contents of the second vial in 99 ml of buffered glycerol contained in a 100 ml ground-glass-stoppered cylinder. Add the dissolved contents of the first vial to this enzyme solution and mix. This reagent is stable for long periods when stored either in a refrigerator or freezer. The freezing point of the reagent is so low that it does not solidify in a freezer.

**Sulfuric acid solution** Cautiously mix 200 ml of concentrated reagent grade sulfuric acid with 1000 ml of distilled water. The solution is stable indefinitely.

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¹Worthington Biochemical Corp., Freehold, New Jersey.
Glucose standard solution, 0.10 mg/ml Dissolve 0.0100 gm of chemically pure glucose in enough saturated benzoic acid solution to make exactly 100 ml of reagent. The solution is stable indefinitely. This standard is equivalent to 100 mg/ml if the 1-10 dilution of blood is used.

Procedure Prepare a 1-10 Somogyi-filtrate by adding 0.2 ml of serum, cerebrospinal fluid or urine to a test tube containing 1.0 ml of water. Add 0.4 ml of 0.3 N barium hydroxide to this and stir exceptionally well. Let stand 5 min and add 0.4 ml of 5% zinc sulfate. Centrifuge or filter.

In a 100 x 16 mm test tube, mix 0.10 ml of a 1:10 Somogyi filtrate of whole blood, heparinized plasma, serum, or cerebrospinal fluid with 1.0 ml of enzyme reagent. (Fluoride, but not thymol, may be used as a preservative.)

A blank and standard containing respectively 0.10 ml of water and 0.10 ml of glucose standard solution are included along with each set of unknowns.

Without delay, incubate all tubes in a 37C water bath for exactly 30 min.

At the end of the incubation period promptly add 5.0 ml of sulfuric acid solution to each tube and mix thoroughly.

Read the pink color of the unknowns and standard at 540 μ against the blank tube. The color is stable for at least 12 hr.
**Calculations**  
This enzymatic procedure does not lend itself to accurate precalibration. Therefore, the following Beer's law formula applies to each unknown.

\[
\frac{\text{Absorbance of unknown}}{\text{Absorbance of standard}} \times 100 = \frac{\text{mg of glucose/100 ml of unknown}}{
\]

**Notes**  
A typical absorbance value for a standard carried through the described procedure is approximately 0.12.

Although it is necessary to include a standard with each set of unknown it is entirely permissible to reuse a single blank tube throughout a given working day.

The time of incubation may be shortened to 15 min in case of emergency, if a standard is incubated for the same length of time. However, the full 30 min period gives more accuracy and precision and should be used routinely.

If a value greater than 450 mg/100 ml is obtained using a 1:10 filtrate and a 30 min incubation period, the procedure must be repeated using a more dilute filtrate. An appropriate correction must be made in the calculations.

**Test for fructose**  
Fructose was determined by copper reduction, employing an adaptation (Harrow *et al.*, 1955) of the Folin-Wu method (1920). Protein-free, Folin-Wu filtrate is heated with an alkaline copper tartrate solution; the fructose reduces the cupric ion to insoluble cuprous compounds. Upon the addition of phosphomolybdic acid reagent, the cuprous compounds dissolve and at the same time reduce the faintly colored phosphomolybdate ion to a lower valence state, yielding densely blue, soluble
oxides of molybdenum. The intensity of the color of the "molybdenum blue" is a measure of the copper reduced to the cuprous condition and therefore of the amount of the fructose present.

**Reagents**

**Alkaline copper tartrate solution**
Dissolve 40 gm of pure anhydrous sodium carbonate in about 400 ml of water and transfer to a liter volumetric flask. Add 7.5 gm of tartaric acid and, when the latter has dissolved, add 4.50 gm of crystalline copper sulfate (CuSO$_4$·5H$_2$O). Mix and dilute to mark. Transfer to a clean, dry, amber bottle.

**Phosphomolybdic acid reagent**
To 35 gm of molybdic acid and 5 gm of sodium tungstate, add 200 ml of 10 per cent sodium hydroxide and 200 ml of water. Boil in a beaker for 30 min, keeping the volume approximately constant by the addition of water from time to time. Cool to room temperature and transfer the solution to a 500 ml volumetric flask. Add 125 ml of concentrated phosphoric acid (syrupy, 85 per cent). Mix, dilute to mark, and transfer to an amber bottle.

**Saturated benzoic acid**
Prepare a saturated aqueous benzoic acid solution.

**Stock 1% fructose solution**
Carefully weigh out 1.0 gm of pure fructose, and transfer to a 100 ml volumetric flask, using saturated benzoic acid solution as solvent. Then dilute to mark with the same solvent. Transfer this solution to a dry, amber bottle. (If crystals of benzoic acid settle out on cooling, ignore them.)
Dilute fructose standard no. 1  Pipette 2.0 ml of the stock 1% fructose solution into a 100 ml volumetric flask. Dilute to mark with saturated benzoic acid solution. Stopper the flask and mix its contents thoroughly. This solution, which should be made up fresh monthly, contains 0.20 mg of fructose per ml.

Dilute fructose standard no. 2  Pipette 4.0 ml of the stock 1% fructose solution into a 100 ml volumetric flask. Dilute to mark with saturated benzoic acid solution. Stopper the flask and mix its contents thoroughly. This solution, which should be made up fresh monthly, contains 0.40 mg of fructose per ml.

Procedure for preparation of a protein-free filtrate (Harrow et al., 1955), an adaptation of the Folin and Wu method (1919)  Pipette 5 ml of sample into a dry 125 ml Erlenmeyer flask. Add 40 ml of N/12 sulfuric acid and mix well. (A burette is convenient for this addition.) Add, drop by drop and with constant shaking of the flask, 5.0 ml of 10% sodium tungstate solution. Stopper and shake vigorously. Allow the flask to stand for 15 min, shaking at 5 min intervals. Have ready a dry, folded filter paper in a dry funnel, beneath which is a dry 125 ml Erlenmeyer flask. Pour a little of the mixture onto the double portion of the filter paper. When the whole paper has become wet, pour the remainder of the mixture into the funnel. Cover the funnel with a watch glass and collect the protein-free filtrate. (It should be perfectly clear and colorless. If the
first small amount of the filtrate is cloudy, it should be refiltered.)

Procedure for fructose determination on protein-free filtrates

Pipette 2.0 ml of the tungstic acid filtrate to a Folin-Wu blood sugar tube. Transfer 2.0 ml of the dilute fructose standard no. 1 to a second blood sugar tube. Add 2.0 ml of alkaline copper tartrate reagent to each tube. (The surface of the mixture should now have reached the middle of the constricted part of the tube.) Mix the contents of the tubes by shaking laterally. Place the tubes in an actively boiling water bath and heat for exactly 6 min. Remove the tubes from the water bath and quickly cool by placing them in a beaker of cold water for 3 min. Add to each tube 2.0 ml of the phosphomolybdic acid reagent. Mix the solutions in the tubes by gentle shaking. (The cuprous oxide should dissolve immediately.) Dilute the contents of the tubes with water to the 25 ml mark. Stopper each tube and mix the contents thoroughly by inverting several times. Compare immediately in a colorimeter. Calculate the number of milligrams of fructose per 100 ml of sample.

Standard curve (Figure 15) Place seven appropriately labeled Folin-Wu sugar tubes in a rack. Pipette into them the quantities of standard fructose solutions and distilled water listed in Table 5.
Figure 15. Standard curve for the determination of fructose (Folin-Wu)
%T O.D. mg %
63.25 0.199  50
35.75 0.447  100
19.50 0.710  150
10.00 1.000  200
 2.75  1.561  300
 0.75  2.000  400

O.D. AT 580 mμ

CONCN. FRUCTOSE (mg %)

50 100 150 200 250 300 350 400
Table 5. Proportional volumes of distilled water and fructose standard solutions used in preparing standard curve for fructose

<table>
<thead>
<tr>
<th>Tube number</th>
<th>ml standard no. 1</th>
<th>ml standard no. 2</th>
<th>ml water</th>
<th>mg per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0</td>
<td>---</td>
<td>2.0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>---</td>
<td>1.5</td>
<td>50</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>---</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>1.5</td>
<td>---</td>
<td>0.5</td>
<td>150</td>
</tr>
<tr>
<td>5</td>
<td>2.0</td>
<td>---</td>
<td>---</td>
<td>200</td>
</tr>
<tr>
<td>6</td>
<td>---</td>
<td>1.5</td>
<td>0.5</td>
<td>300</td>
</tr>
<tr>
<td>7</td>
<td>---</td>
<td>2.0</td>
<td>---</td>
<td>400</td>
</tr>
</tbody>
</table>

Add 2 ml of alkaline copper tartrate reagent to each tube. (The surface of the mixture should now have reached the middle of the constricted part of the tube.) Mix the contents of the tubes by shaking laterally. Place the tubes in actively boiling water and heat for exactly 6 min. Remove the tubes and cool them at the same time by placing them in a beaker of cold water for 3 min. Add to each tube 2.0 ml of the phosphomolybdic acid reagent. Mix the solutions in the tubes by gentle shaking. (The cuprous oxide should dissolve immediately.) Dilute the solutions in the tubes with water to the 25 ml mark. Stopper each tube and mix the contents thoroughly by inverting several times. Read in the photoelectric colorimeter within 10 min.
using the appropriate filter. One with maximal transmission in the orange at 580 μm will give good results. Prepare a calibration curve on linear or semilog graph paper by plotting the data.

Test for the amount of glucose and fructose consumed by *C. thermocellum* Glucose and fructose determinations were made on 12 day old D58 broth cultures of *C. thermocellum* strains TET, 157 and 651.

**Preparation of Medium D58 for Turbidometric Studies**

D58 medium was prepared, autoclaved and filter sterilized (Millipore; 0.45 micron porosity). The preparatory autoclaving was done to remove high temperature precipitable material which would ordinarily interfere with turbidometric studies. One-hundred ml volumes of this medium were dispensed aseptically into 6-ounce prescription bottles, to each of which were added filter sterilized carbohydrates and sodium thioglycollate. Each culture bottle was inoculated with 2.5 ml of an actively growing D-58 + cellobiose-grown, broth culture of *C. thermocellum* (157) of 0.25 optical density at 340 μm wavelength. These cultures were then incubated for 60 hr. Periodically, optical density readings on aliquots of these cultures were made at 340 μm wavelength on a Bausch & Lomb Spectronic 20 colorimeter.
Conditions in Hungate's Medium Which Influence the Rates of Growth and Utilization of Hexoses by C. thermocellum

Experiment no. 1: Effect of yeast extract

Hungate's medium was employed in this experiment, while one portion of the medium was modified to contain a yeast extract concentration of 0.45%, instead of the usual 0.05%. One-hundred ml volumes of this medium were dispensed aseptically into 6-ounce prescription bottles, to each of which were added filter-sterilized carbohydrates as before. Each culture bottle was inoculated with 2.5 ml of an actively growing, cellobiose-grown, broth culture of C. thermocellum (157) of 0.25 optical density at 340 μm wavelength; the cultures were incubated for 72 hr. Periodically, optical density readings on aliquots of these cultures were made at 340 μm wavelength on a Bausch & Lomb Spectronic 20 colorimeter.

Experiment no. 2: Effect of asparagine

Filter-sterilized asparagine was added aseptically to Hungate's medium to give a final concentration of 23 mg%. Filter-sterilized glucose and cellobiose were added aseptically to 16 x 125 mm screw-capped tubes, each of which contained 6 ml of the test medium. Broth tubes containing no added asparagine were employed as controls. The tubes of broth medium were inoculated with 0.05 ml of a 48 hr, cellobiose-grown, broth culture and were incubated for a 10 day period.
Experiment no. 3: Effect of glutamine

Experiment no. 2 was repeated, substituting glutamine for asparagine at a final concentration of 30 mg%.

Experiment no. 4: Effect of biotin

Employing the foregoing experimental procedures (Expts. no. 2 and 3), biotin was substituted as the growth factor to a final concentration of 16 μg/L.

Experiment no. 5: Effect of heat-treated glucose

Each of four 8-ounce prescription bottles was filled to 100 ml volume with HMM. Glucose was added to one of the four culture bottles prior to autoclaving at 15# steam pressure and 121°C. Following the autoclaving procedure, the second and third bottle received filter-sterilized and heat-treated glucose, respectively. The heat-treated glucose consisted of a distilled water-glucose mixture which had been autoclaved for 15 min at 15# steam pressure and 121°C. The fourth bottle received no carbohydrate and served as the carbohydrate-free control. The culture bottles were inoculated with 2.5 ml of an actively growing culture of C. thermocellum of 0.25 optical density at 340 μm wavelength, and were incubated for 110 hr. Periodically, optical density readings on aliquots of these cultures were made at 340 μm wavelength on a Bausch & Lomb Spectronic 20 colorimeter.
Experiment no. 6: Effect of heat-treated cellobiose

A modification of experiment no. 5 was then setup substituting cellobiose for glucose as the principal carbohydrate source. Culture bottles were inoculated with 2.5 ml of an actively growing culture of *C. thermocellum* (157) of 0.25 optical density at 340 μm wavelength, and incubated for 110 hr. Periodically, optical density readings on aliquots of these cultures were made at 340 μm wavelength on a Bausch & Lomb Spectronic 20 colorimeter.

Comparative Growth Response of *C. thermocellum* to Cellobiose, Glucose and Fructose

Procedure for log phase, cellobiose-grown, mother culture employed as inoculum

One-hundred ml volumes of Hungate's modified medium were dispensed aseptically into 6-ounce prescription bottles, to each of which were added filter-sterilized carbohydrates. Each culture bottle was inoculated with 2.5 ml of an actively growing, cellobiose-grown, broth culture of *C. thermocellum* of 0.25 optical density readings at 340 μm wavelength; the culture bottles were incubated for 8 days. Daily optical density readings on aliquots of these cultures were made at 340 μm wavelength on a Bausch & Lomb Spectronic 20 colorimeter.
Procedure for Chromatographic Analysis for Cellobiose, or Cellodextrins, in Cell-Free Extracts of C. thermocellum

Filter-sterilized glucose was added to nine 8-ounce prescription bottles, each of which contained 100 ml volumes of Hungate's modified medium. These culture bottles were then inoculated with an actively growing culture of C. thermocellum (157), and incubated for about 60 hr. Upon reaching the exponential phase of growth (O.D. of 0.2291 or 59%T) these cultures were centrifuged at 16,300 x g for 30 min in a Sorvall Super-speed centrifuge, model RC-2. The cells were twice washed in saline and centrifuged at 27,000 x g for a 30 min period. The packed cells were resuspended in 3 ml of distilled water, frozen (at -20C) and thawed (at 25C) five times, followed by 5 min of sonication (Biosonik; Bronwill Scientific, Rochester, N.Y.). (The cells were completely disrupted by this treatment as evidenced by appearance on microscopic examination of a smear of the preparation.) The disrupted cell suspension was centrifuged at 27,000 x g for 30 min to remove cellular debris. The supernatant material was retained for chromatographic analysis according to the methods of Ke and Quinn (1965).

Experiment no. 1: Procedure for growth response of C. thermocellum to a mixture of glucose and α-D-glucose-1-phosphate

D-58 broth was prepared and distributed aseptically into each of fifteen 16 x 125 mm screw-capped tubes. Filter-sterilized sodium thioglycollate was added aseptically to each
tube to give a final concentration of 0.04%. These tubes were arranged in a series of triplicate tubes. The first, second, third, and fourth series received equimolar \((8.33 \times 10^{-3} \text{M})\) concentration of filter-sterilized glucose, \(\alpha\-D\-glucose\-l\-phosphate\), glucose and cellobiose, respectively. To one of the glucose-containing series, an equimolar concentration of \(\alpha\-D\-glucose\-l\-phosphate\) (Sigma Chem. Co.; St. Louis, Mo.) was added aseptically. The fifth series of three tubes received no added carbohydrate and served as the carbohydrate-free controls for this experiment. All culture tubes were inoculated with 0.1 ml of a 2 week old, cellobiose-grown, mother culture of \(C. \text{thermo}-\text{cellum}\) (157), and were incubated for 48 hr. After 48 hr of incubation, optical density readings of the cultures were made at 340 \(\mu\) wavelength on a Bausch & Lomb Spectronic 20 colorimeter.

**Experiment no. 2: Procedure for growth response of \(C. \text{thermo}-\text{cellum}\) to a mixture of glucose and \(\alpha\-D\-glucose\-l\-phosphate\)**

The foregoing experiment (Expt. no. 1) was repeated employing Hungate's modified formula as the culture medium.

**Procedure for Growth Response of \(C. \text{thermo}-\text{cellum}\) to Cellobiose, Glucose and Fructose When Using Inocula of Different Physiological States-I**

**Experiment no. 1: First passage, glucose-grown, log phase mother culture employed as inoculum**

One-hundred ml volumes of Hungate's modified medium were dispensed aseptically into 6-ounce prescription bottles, to
each of which were added filter sterilized carbohydrates. Each culture bottle was inoculated with 2.5 ml of a glucose-grown, log phase, mother culture; the culture bottles were then incubated for 61 hr. Periodically, optical density readings on aliquots of these cultures were made at 340 µm wavelength on a Bausch & Lomb Spectronic 20 colorimeter.

Experiment no. 2: Procedure with first passage, fructose-grown, log phase mother culture

One-hundred ml volumes of Hungate's modified medium were dispensed aseptically into 6-ounce prescription bottles, to each of which were added filter sterilized carbohydrates. Each culture bottle was inoculated with 2.5 ml of a fructose-grown, log phase, mother culture; the culture bottles were then incubated for 61 hr. Periodically, optical density readings on aliquots of these cultures were made at 340 µm wavelength on a Bausch & Lomb Spectronic 20 colorimeter.

Procedure for Chromatographic Analysis for Cellobiose, or Cellodextrins, in Glucose and Fructose Broth Culture Filtrates

Filter-sterilized glucose was added to six of a series of twelve 8-ounce prescription bottles, each of which contained 100 ml volumes of Hungate's modified medium. The remaining six culture bottles received equivalent amounts of filter-sterilized fructose. The culture bottles were then inoculated with 2.5 ml of a 48 hr culture of C. thermocellum (157), and were incubated.
Upon reaching the logarithmic phase of growth, three of each of these glucose and fructose culture bottles were removed from incubation. When the remaining six culture bottles had reached the stationary phase of growth, they too were removed from incubation. The cells were harvested by centrifugation at 16,300 x g for 30 min in a Sorvall Superspeed centrifuge, model RC-2. Five ml samples were taken from each of the four different culture filtrates so obtained, and these aliquots were retained for chromatographic analysis. The remaining filtrate volumes were placed separately in four 9" x 13" pyrex glass baking dishes and were evaporated to dryness at 60°C (about 24 hr were required). Each of the four dried samples was taken up (for extraction of sugars) in 20 ml of a butanol, ethanol, water-mixture composed of a concentration-ratio of five:four:one, respectively. The butanol extracts of the dried filtrates were allowed to settle, then the supernates were retained. Each of the four supernates was evaporated to dryness and then were taken up in 3 ml of distilled water. All of the resulting aqueous extracts of filtrates were then analyzed chromatographically for the presence of cellobiose, or larger oligosaccharides, employing the methods of Ke and Quinn (1965).
Procedure for Test of Growth Response of *C. thermocellum* to Cellobiose, Glucose and Fructose When Employing Inocula of Different Physiological States-II

Experiment no. 1: Procedure with fifth-serial-passaged, glucose-grown, mother culture employed as inoculum

One-hundred ml volumes of Hungate's modified medium were dispensed aseptically into 6-ounce prescription bottles, to each of which were added filter sterilized carbohydrates. Each culture bottle was inoculated with 2.5 ml of a fifth-serial-passaged, glucose-grown, mother culture; the culture bottles were incubated for a period of 62 hr. Periodically optical density readings on aliquots of these cultures were made at 340 μm wavelength on a Bausch & Lomb Spectronic 20 colorimeter.

Experiment no. 2: Procedure with fifth-serial-passaged, fructose-grown, mother culture employed as inoculum

One-hundred ml volumes of Hungate's modified medium were dispensed aseptically into 6-ounce prescription bottles, to each of which were added filter-sterilized carbohydrates. Each culture bottle was inoculated with 2.5 ml of a fifth-serial-passaged, fructose-grown, mother culture; the culture bottles were incubated for 50 hr. Periodically, optical density readings on aliquots of these cultures were made at 340 μm wavelength on a Bausch & Lomb Spectronic 20 colorimeter.
Evidence for Inductive Permeation as an Explanation for the Delayed Lag Phase Shown of Cultures Grown in Glucose Broth

Procedure for permeation studies with $^{14}C$-glucose

*C. thermocellum* (157) was cultivated in two 8-ounce prescription bottles containing 100 ml volumes of Hungate's modified medium. One bottle of culture medium received glucose, while the other received cellobiose. Each culture bottle was inoculated with 2.5 ml of an actively growing, cellobiose-grown, mother culture, and was incubated. Upon reaching the exponential phase of growth, cells were collected by centrifugation at 27,000 $x$ g for 20 min in a Sorvall Centrifuge, model SS3 (Ivan Sorvall, Inc., Norwalk, Connecticut). Cells from the cellobiose-grown culture were centrifugally washed twice in normal saline (0.85%) by centrifuging at 27,000 $x$ g for 20 min. These washed cells were then taken up in carbohydrate-free basal medium (HMM) to give a final cell density of 0.3 O.D. units. Two 10 ml aliquots were placed in each of two 16 x 150 mm screw-capped tubes. One tube received UL-$^{14}C$-glucose (Int. Chem. & Nuclear Corp.; City of Industry, Calif.) to give a final concentration of 1 μ Curie per ml, while the second tube received no added carbohydrate and served as the radioactivity-free control. Both tubes were immediately placed in a 60°C waterbath for 1 hr (incubation was done aerobically). Following this incubation, the cells were centrifuged at 4,600 $x$ g for 30 min (Sorvall Angle Centrifuge) and the supernatant fluid was
decanted. The cells were twice resuspended in 10 ml of normal saline after centrifugation at 4,600 x g for 30 min periods. Following the second washing and centrifugation step, the cell pellet was resuspended in 10 ml of scintillation fluid of the following composition (gm/L): Naphthaline, 100 gm (Baker; Chicago, Illinois); 1,4-Dioxane, 1 Liter (Baker); 2,5-Diphenyloxazole, 5 gm (Beckman; Fullerton, Calif.). One ml of the above cell suspension was added to a scintillator vial containing 9 ml of scintillation fluid. The radioisotope-free cell control was treated in like manner. The two vials were placed into a liquid scintillator counter (Beckman DPM-100) and measured for radioactivity.

Upon reaching the exponential phase of growth, the glucose-grown cells were treated as above and were examined for radioactivity.

Procedure for permeation studies with $^{14}$C-fructose

*C. thermocellum* (157) was cultivated in two 8-ounce prescription bottles containing 100 ml volumes of Hungate's modified medium. One bottle of culture medium was supplemented with fructose, while the other received cellobiose. Each culture bottle was inoculated with 2.5 ml of an actively growing, cellobiose-grown, mother culture and was incubated. Upon reaching the exponential phase of growth, cells were collected by centrifugation at 27,000 x g for 20 min. The cellobiose-grown cells were twice resuspended in 10 ml of normal saline followed by
centrifugation at 27,000 x g for 20 min. These washed cells were then taken up in carbohydrate-free medium (HMM) to give a cell density of 0.3 O.D. units. Two 10 ml aliquots were placed in each of two 16 x 150 mm screw-capped tubes. One tube received 0.02 ml of UL-C\textsuperscript{14}-fructose (Int. Chem. and Nuclear Corp.; City of Industry, Calif.) to give a final concentration of 1 mCi per ml, while the second tube received no carbohydrate and served as the radioactivity-free control. The two tubes were placed in a 60°C waterbath for 1 hr (incubation was done aerobically). Following incubation, the cells were centrifuged at 4,600 x g for 30 min (Sorvall Angle Centrifuge) and the supernatant fluid was decanted. The packed cells were twice suspended in 10 ml of normal saline after being centrifuged at 4,600 x g for 30 min periods. Following the second washing and centrifugation, the cell pellet was resuspended in a 10 ml volume of scintillation fluid. One ml of the resultant cell suspension was added to a scintillator vial containing 9 ml of scintillation fluid. The radioisotope-free cell control was treated in like manner. The two vials were placed into a liquid scintillator counter, and measured for radioactivity.

Upon reaching the exponential phase of growth, the fructose-grown cells were treated as above and were also examined for radioactivity.
RESULTS AND DISCUSSION

Conditions in Medium D58 Which Influence the Growth and Utilization of Hexoses by \textit{Clostridium thermocellum}

\textbf{Effect of Zymosan}

Since medium D58 readily supports the utilization of cellulose and cellobiose by \textit{C. thermocellum}, and since this medium had been worked with and formulated by a member of our group (Quinn, 1949), it was selected as the medium of choice for studies of the three strains of \textit{C. thermocellum}. The problem was begun by inoculating the three strains of \textit{C. thermocellum} into D58-glucose broths. It was noted that an occasional tube of glucose-containing broth gave rise to growth, while almost invariably the cellobiose-containing broth tubes supported growth; growth was always supported when cellulose was employed as the carbohydrate source. The number of inoculated D58-glucose broths giving rise to growth appeared to occur too frequently to attribute to a mutational event. Furthermore, it seemed that if a mutation were responsible for this growth it could be easily perpetuated by continued transfers into glucose-containing medium; however, when glucose-grown cultures were returned to glucose broth, they usually failed to grow, and thus behaved no differently than culture tubes inoculated with a cellobiose-grown mother culture.

At this point there was ample reason to be optimistic: an occasional glucose broth gave rise to growth, and the condition
did not appear to be the result of mutation. But why the erratic, unconventional behavior? If not mutation, then what? These were the obvious questions. So with no more to go by than cold intuition for direction, the "shotgun approach" to research was adopted: first one thing was tried and then another.

It was reported (Quinn, 1949; Beers, et al., 1969) that yeast extract was the richest source of factors for growth and cellulose digestion by a microaerophilic, cellulolytic bacterial culture. These reports were responsible for the consideration and introduction into our experiments of a substance called Zymosan: a purified, insoluble yeast cell wall material composed chemically of glucose, glucosamine and mannose (Cseh and Szabo, 1961). A measured amount of this material was mixed with D58 broth prior to heat sterilization. Following sterilization, filter sterilized glucose was added aseptically to the Zymosan-containing D58 medium. The tubes were inoculated and incubated in the usual manner. To our amazement, glucose was vigorously fermented in the Zymosan-containing culture tubes, while the broths which contained only Zymosan showed no growth; and as usual, only an occasional broth, containing only glucose as the sugar source, supported growth.

Next, fructose was introduced into the above experimental procedure, and it too behaved like glucose in the presence of Zymosan; but the fermentation of fructose in the absence of Zymosan was never observed.
Despite these observations we were left in a quandary concerning the functional role of Zymosan. Unfortunately, the work with Zymosan did not progress any further, but continued efforts to understand the hexose problem were pursued along other avenues.

Effect of heated glucose

Stanier (1942a, b) reported that heated glucose was toxic to certain members of the genus Cytophaga, a group of cellulose-digesting bacteria. This report, together with other reports scattered throughout the literature concerning the nature of heated glucose, caused most investigators working with cellulolytic bacteria to resort to the use of filter sterilized carbohydrates. But despite the reports on toxicity of heated glucose, several reports were encountered in the literature which described heated glucose as being stimulatory to certain species of the genus Bacillus. These reports provoked some curiosity, and experiments were conducted which involved the addition of glucose to medium D58, prior to heat sterilization. The culture tubes were inoculated, together with proper controls (e.g., D58 broth medium with filter sterilized glucose), and incubated for several days. The results of this experiment showed that heated glucose promoted growth comparable to that seen with the Zymosan-glucose combination.

Experiments were immediately conducted using glucose which had been heated in the absence of the growth medium. It, too,
had a growth promoting effect.

**Effect of reducing agent**

At about the time that the heated glucose experiments were in progress, it was observed that, for some unknown reason, a number of the cellobiose-containing culture tubes failed to show growth. A few other such incidents were all that was needed to suspect inadequate anaerobiosis as a cause of growth failure—and to consider that this condition perhaps also was related to the glucose and fructose problem. Therefore, in the following experiments, filter-sterilized sodium thioglycollate (a reducing agent) was prepared and added aseptically to all of the culture tubes. The results of those experiments revealed that the erratic growth behavior previously encountered with broths containing filter sterilized glucose, fructose and cellobiose was attributable to inadequate anaerobiosis. And although organisms grown in medium containing the soluble carbohydrates required an initial lower oxidation-reduction potential to prevent erratic growth patterns, those cultures inoculated into D58-cellulose broth did not.

The ability of the organisms to grow more readily in cellulose-containing broth, in the absence of a reducing agent, is probably due to the ability of the organism to attach itself onto (or within) the cellulose fibers, and hence being at a broth level that is less exposed to possible traces of oxygen.
Although the specific function of Zymosan in promoting glucose and fructose fermentation was not elucidated, it is speculated that it, too, functions as an anchor (or attachment site) in a manner similar to that of cellulose, thus affording the organisms a greater opportunity to grow in a more reduced environment; or with normal substrate present a reductase system may operate.

In part, the objective of research on the glucose problem had been achieved: It was shown that *C. thermocellum* does, in fact, ferment glucose; and two of the three strains also fermented fructose; strain 651 did not, however, appear to utilize fructose. The observed results did not appear to be the consequence of mutation since, when old stock cultures that had not at any time been grown in hexose sugars, were exposed to glucose and fructose under reduced oxidation-reduction potential, they responded similarly to the current working cultures; and when the glucose and fructose-grown cultures were returned to sodium thioglycollate-free broth, they failed to grow, whereas the same cultures inoculated into cellobiose-containing broths were able to grow.

**Carbohydrate Determinations**

**Rate of fermentation of glucose and fructose by *C. thermocellum***

The amount of glucose and fructose consumed by the three strains of *C. thermocellum* was assayed via the Glucose Oxidase
Test for glucose, and the copper reduction test (Folin-Wu) for fructose. These results are tabulated in Table 6.

Preparation of D58 Medium for Turbidometric Studies of Cellobiose and Glucose Utilization

Although glucose and fructose were fermented by \textit{C. thermocellum}, there appeared to be a consistent delayed growth response for the utilization of these sugars, sometimes lasting for as long as a week in the case of fructose. In order to obtain a clearer picture of the growth response shown by the organisms for the various carbohydrates, a growth curve experiment was conducted in D58 broth (Figure 16). It may be noted that cellobiose is readily fermented with only a slight lag; and although good growth is obtained in the glucose-containing broth, a marked lag is observed.

Conditions in Hungate's Medium Which Influence the Rates of Growth and Utilization of Hexoses by \textit{Clostridium thermocellum}

Effect of yeast extract

It seemed somewhat ironic, that up to this point we had managed to determine that the organism was actually capable of fermenting glucose and fructose, but without knowing why. So, as mentioned earlier, one objective of the experiment had been attained. The next aspect of the problem was quite obvious: Why did D58 and not Hungate's medium support the fermentation of glucose and fructose? A pursuit in that direction commenced by preparing Hungate's medium and running the routine tests to
Table 6. Rate of glucose and fructose fermentation by three strains of Clostridium thermocellum

<table>
<thead>
<tr>
<th>Clostridium thermocellum</th>
<th>Per cent of added sugar consumed in 12 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>glucose</td>
</tr>
<tr>
<td>Strain TET</td>
<td>90</td>
</tr>
<tr>
<td>Strain 157</td>
<td>84</td>
</tr>
<tr>
<td>Strain 651</td>
<td>40</td>
</tr>
</tbody>
</table>

determine whether or not this medium would support the fermentation of glucose and fructose by the three strains of C. thermocellum. The results were that, while growth was obtained in the cellobiose-containing broths, the glucose and fructose broth tubes failed to show growth.

The next logical approach was to determine what factor(s) in medium D58 was responsible for permitting glucose and fructose to be fermented. However, in this nutritional study, no small measure of difficulty was encountered because of the amount of precipitate that forms and is lost from solution during medium D58 preparation and heat sterilization. If the medium were filter- rather than heat-sterilized, large amounts of precipitate still continued to form at thermophilic incubation temperatures, making the problem no less difficult. Our first thoughts were to determine qualitatively and quantitatively
Figure 16. Growth of *C. thermocellum* (157) in glucose and cellobiose-containing D58 medium.
the factors in the medium that were being lost in the precipitate.

Man, being the lazy creature that he is, must seek the easier approach to all problems; so typically, I first took one step backward, and reconsidered the whole matter. After a careful look at the composition of the two media (D58 and Hungate's), one prominent feature became apparent: medium D58 was considerably darker in color than Hungate's medium. There could be only one substance responsible for this color difference, and that was yeast extract. Upon observing the formulae of the two media (Table 1 & 4), one finds that D58 contains a 0.45\% concentration of yeast extract, whereas Hungate's formula contains only 0.05\%.

This observation of color difference prompted experiments directed at determining whether yeast extract really was the determining factor responsible for the fermentation of glucose and fructose by \textit{C. thermocellum}. Hungate's medium was prepared and supplemented with yeast extract to give a final concentration of 0.45\%. One observes (Figure 17) a three-fold effect when 0.45\% yeast extract is used in Hungate's medium: (1) A decrease in the lag phase time for the utilization of cellobiose; (2) A denser cell population—presumably due to more efficient fermentation of cellobiose; and (3) most importantly, that glucose is fermented; however, a distinct preference for cellobiose and a marked lag phase for glucose is noted.
Figure 17. Growth of *C. thermocellum* (157) in Hungate's medium which contains both normal (0.05%) and elevated (0.45%) concentrations of yeast extract.
© BASAL MEDIUM (B.M.) WITHOUT CARBOHYDRATE

X B.M. + CELLOBIOSE + 0.45 % YEAST EXTRACT

■ B.M. + CELLOBIOSE + 0.05 % YEAST EXTRACT

▲ B.M. + GLUCOSE + 0.45 % YEAST EXTRACT

O.D. AT 340 nm

HOURS
McBee's (1950) reason for using such meager amounts of yeast extract in his studies (with Hungate's medium) was to reduce the possible complications of additional carbon in medium employed for fermentation balance studies. He apparently considered the possible beneficial effects of additional growth factors, as indicated by the following statement: "All of the cultures grow well in a mineral-base medium containing cellulose and a trace of yeast extract (0.05 per cent). This growth is not enhanced by larger amounts of yeast extract or by the addition of peptones, serum, plant juices, or extracts of soil or manure." Obviously those comments must have been made with reference to medium containing cellulose as the carbohydrate source, since all of his efforts to elucidate the reason behind the organism's inability to ferment glucose, employed medium containing a 0.05% concentration of yeast extract.

It was now known that yeast extract was the significant growth factor(s) needed for the utilization of glucose and fructose; but as with any research, one question invariably leads to another, as indicated in the following section:

**Effect of asparagine**

The next interesting problem to develop (but a seemingly overwhelming one, when one considers the nature of yeast extract) was the one determining the specific factor(s) in yeast extract effecting this behavior toward hexoses—if any single specific factor even exists. The yeast extract may simply provide a
general over-all high nutritional level necessary to favorably effect the preparatory conditions for glucose and fructose utilization.

The possible role of asparagine in hexose metabolism by C. *thermocellum* was considered, since it appeared to enhance the utilization of glucose by washed cell preparations of *P. freudenrichii* (Field and Lichstein, 1958b). Therefore asparagine was tested, but it provided no growth promoting effect (as shown in Table 7) with glucose (or cellobiose) as the carbohydrate source in Hungate's medium.

**Effect of glutamine**

Because of the reports describing the stimulatory effects of glutamine on glucose fermentation by *C. tetani* (Lerner and Mueller, 1949) and hemolytic streptococci (McIlwain, 1946), an experiment was conducted which employed Hungate's medium supplemented with this compound. The results, however, indicated no stimulatory effect, as evidenced by failure of the organisms to grow in medium supplemented with this amino acid.

**Effect of biotin**

Since yeast extract is a rich source of biotin, and since biotin enhances the fermentation of cellulose by *C. thermocellulaseum* (Enebo, 1954), it was tested as a possible stimulatory agent in glucose fermentation by *C. thermocellum* in Hungate's standard (or minimal) medium. Biotin did not, however, appear
Table 7. The influence of asparagine on glucose and cellobiose utilization by *C. thermocellum* (157). Observations were made after 10 days of incubation^a^.

<table>
<thead>
<tr>
<th>No. of tubes</th>
<th>Medium</th>
<th>Carbohydrate</th>
<th>Asparagine added</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Hungate's</td>
<td>glucose</td>
<td>yes</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>&quot;</td>
<td>glucose</td>
<td>no</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>&quot;</td>
<td>cellobiose</td>
<td>yes</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>&quot;</td>
<td>cellobiose</td>
<td>no</td>
<td>+</td>
</tr>
</tbody>
</table>

^a^Key to notation: - denotes no growth  
+ denotes good growth.

to promote growth in medium containing glucose as the carbohydrate source.

**Effect of heated carbohydrates**

Since concern was with discovery of growth promoting agents which might be effective in enhancing the fermentation of hexose sugars, attention next was turned to the topic dealing with growth stimulation of microorganisms by heated carbohydrates. This area of study had but briefly been looked at, and had not been examined either quantitatively or qualitatively with the present working medium (Hungate's). A review of the literature revealed that an enormous amount of work had been done on the subject of growth stimulation of microorganisms by heated carbohydrates, dating as far back as 1927 (Fulmer and Heussleman) to a more recent publication by Coulter* et al.* (1966).
Experiments were conducted with heat treated glucose and cellobiose, together with filter sterilized sugars serving as controls. It will be noted (Figure 18) that glucose, heated either in distilled water or together with the medium, is considerably more stimulatory than the filter sterilized preparation. The same pattern of stimulation is observed with cellobiose (Figure 19). One may note, too, that heated sugars enhance growth but that the over-all cell density, as compared with that of unheated medium (Figures 17, 20, 21, 22, 23, 24) was reduced and that the lag phase was extended. Unlike heated D58 medium (Figure 16), Hungate's medium is apparently impaired by autoclaving.

Ramsey and Lankford (1956) reported the stimulatory effect on several types of bacteria, of glucose heated as a component of the complete medium, together with phosphate, or heated alone. The stimulatory effect of glucose heated together with phosphate was found to be superior to that of glucose autoclaved together with the medium base, of glucose autoclaved alone, or of the filter sterilized preparation. Glucose that was autoclaved in the absence of the growth medium, or of phosphate, was the least stimulatory of the four glucose preparations. It was also found that the stimulatory agent(s) from the heated glucose-phosphate mixture was ether soluble; however, the author stated that "the compound(s) has not been identified but does not appear to be an aldehyde or ketone, a phosphorylated derivative of glucose, a volatile compound, a reducing
Figure 18. A comparison of the growth-promoting effect on C. thermocellum (157) of heat-treated and filter-sterilized glucose in Hungate's modified medium
- BASAL MEDIUM (B.M.) WITHOUT CARBOHYDRATE
- GLUCOSE (AUTOCLAVED WITH B.M.)
- GLUCOSE (AUTOCLAVED SEPARATELY)
- GLUCOSE (FILTER STERILIZED)
Figure 19. A comparison of the growth-promoting effect on *C. thermocellum* (157) of heat-treated and filter sterilized cellobiose in Hungate's modified medium.
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0

O.D. AT 340 m\(\mu\)

- ○ BASAL MEDIUM (B.M.) WITHOUT CARBOHYDRATE
- □ CELLOBIOSE (AUTOCLAVED WITH B.M.)
- ◇ CELLOBIOSE (AUTOCLAVED SEPARATELY)
- △ CELLOBIOSE (FILTER STERILIZED)

0 10 20 30 40 50 60 70 80 90 100 110

HOURS
agent, or an acidic compound."

Lankford et al. (1957) tested a variety of chemical compounds for their capacity to substitute for autoclaved glucose-phosphate solutions in stimulation of growth initiation of Bacillus globigii. He postulated that glucose phosphate, and substances which substitute for it, function as specific chelating agents to supply the cell with metals essential to cell division and growth, in a nontoxic, assimilable, form.

Field and Lichstein (1958a) have reported work dealing with the growth stimulating effect of autoclaved glucose media and its relationship to the CO₂ requirement of Propionibacterium freudenreichii. Their data led them to suggest that reducing carbohydrates, such as glucose, react with phosphates and amino acids during heating to produce a factor(s) which replaces the CO₂ requirement for initiation of growth from small inocula. Glucose, which was autoclaved in the absence of the medium, failed to support growth.

The studies in this investigation with heat treated carbohydrates were of a cursory nature, and do not warrant stringent comparisons to the detailed studies of the foregoing reports. Superficially and in general, however, the growth responses by C. thermocellum appear to be similar to those of these other species, but with some notable differences. In our studies it was observed that glucose, when autoclaved in the absence of the growth medium base and then added aseptically, was markedly stimulatory. In their studies, this preparation of glucose
provided no stimulatory effects, but instead was more toxic than even the filter-sterilized glucose. The nature of the compounds(s) formed from a heated-distilled water-glucose mixture, or the nature of its stimulatory effects were not determined, since this aspect of the present investigation was only of a temporary interest and was not pursued any further.

Comparative Growth Response of *C. thermocellum* to Cellobiose, Glucose and Fructose

At this point, the investigator returned to work on the main course of this investigation, which was to study (among other things) the utilization of normal, non-heated, carbohydrates under normal growth conditions. It will be recalled that fructose, as well as glucose, was utilized when Hungate's standard growth medium was supplemented with a concentration of 0.45% yeast extract; and although an extended lag phase was observed for growth in medium containing glucose as the principal carbohydrate source, there appeared to be greatly delayed growth, even beyond that seen for glucose, when the organisms were inoculated into fructose broth.

At this point, an experiment was conducted in which growth in the various carbohydrate broths was measured on a daily basis (instead of hourly), since production of visible growth in fructose broth normally required days. The results of that experiment are as seen in Figure 20. Here one may see that fructose is utilized, but only after a lag period which goes
Figure 20. Comparative growth response of *C. thermocellum* (157) to cellobiose, glucose and fructose in Hungate's modified medium.
O BASAL MEDIUM (B.M.) WITHOUT CARBOHYDRATE
X B.M. + CELLOBIOSE + 0.45% YEAST EXTRACT
△ B.M. + GLUCOSE + 0.45% YEAST EXTRACT
○ B.M. + FRUCTOSE + 0.45% YEAST EXTRACT

O.D. AT 340 mµ

0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0

0 1 2 3 4 5 6 7 8

DAYS
even beyond that required for observable growth with glucose. The eventual growth rate, as well as the maximum cell density produced, appear to be somewhat similar with both carbohydrates. Mutation would not appear to be an explanation for the extended lag phase seen with cultures grown in fructose broth, since the cultures do not grow when returned to broths containing low levels of yeast extract (< 0.05%), a concentration at which cellobiose and cellulose are fermented. In this respect, therefore, the cultures behave as they normally would, were the inoculum a cellobiose-grown mother culture.

From analysis of the growth patterns observed in Figure 6, four hypotheses were formulated to explain the delayed growth response shown for glucose and fructose-grown cultures; these are listed below in order of preference:

1. Cellobiose must first be formed within the cell before growth commences; (2) the cells must undergo some physiological adjustment (e.g., transport induction) before glucose and fructose can be taken up by the cell; (3) cellobiose must be formed at the cell surface prior to being metabolized; (4) the cells, after a period of physiological adjustment, elaborate an extracellular enzyme into the growth medium which catalyzes the formation of cellobiose, or cellodextrins, from glucose or fructose. The next series of experiments were devised to test these hypotheses.
Chromatographic Analyses for Cellobiose, or Cellodextrins, in Cell-Free Extracts of \textit{C. thermocellum}

Cellobiose phosphorylase, an intracellular enzyme which catalyzes the \textit{in vitro} formation of cellobiose from a mixture of D-glucose and \(\alpha\)-D-glucose-1-phosphate, has been demonstrated for \textit{C. thermocellum} (Sih and McBee, 1955; Sih \textit{et al.}, 1957; Alexander, 1961, 1968a, b). Therefore, deriving from this information, the first hypothesis was proposed: Cellobiose must first be formed within the cell before growth commences. At first glance this hypothesis appeared to be the most logical and most appealing one of the four, and hence was the first to be tested. An experiment involving chromatographic analysis of cell-free extracts from glucose-grown cells was conducted. The results of this experiment revealed the absence of cellobiose (or larger oligosaccharides) in the cell-free extracts. Also of interest was the observation that glucose, the principal energy source on which the cells were grown, was also absent.

Hypothesis \#2 (which states that the cell must undergo some physiological adjustment—e.g., transport induction—before glucose and fructose can be taken up by the cell), due to certain technical difficulties, was deferred until last, \textit{q.v.}
Growth Response of C. thermocellum to a Mixture of Glucose and α-D-glucose-1-phosphate

Hypothesis #3 (cellobiose is formed at the cell surface prior to being metabolized) was tested next by an experiment which involved growing the organisms in broth containing a mixture of glucose and α-D-glucose-1-phosphate. It was felt that if a cell surface enzyme existed which was capable of converting two molecules of glucose to a molecule of cellobiose, before carbohydrate could enter the cell, the time required for this conversion step might be measurably decreased if one of the molecules of glucose were supplied in an already converted form (e.g., α-D-glucose-1-P). To this end, two experiments were conducted: one employing medium D58 and the other, Hungate's modified medium. The results (Table 8 & 9) did not appear to indicate a decrease in time required for growth; if anything, the α-D-glucose-1-phosphate was slightly inhibitory.

Growth Response of C. thermocellum to Glucose and Fructose When Employing Inocula of Different Physiological States-I

Results with first passage, glucose-grown, log phase mother culture employed as inoculum

Hypothesis #4 stated that the cells, after a period of physiological adjustment, elaborate an extracellular enzyme into the growth medium which catalyzes the formation of cellobiose, or celloextrins, from glucose or fructose. It was speculated that if hypothesis #4 were correct, one might expect
Table 8. Data from an experiment designed to determine whether a combination of α-D-glucose-1-phosphate and glucose would reduce the extended lag phase normally exhibited for the growth of *C. thermocellum* (157) in glucose-containing broth. The results are for D58 medium, and are interpreted as the mean of the per cent transmittance and optical density of three culture tubes within each of the five treatments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>%T₁</th>
<th>%T₂</th>
<th>%T₃</th>
<th>X%T</th>
<th>X O.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>52.5</td>
<td>50.0</td>
<td>48.0</td>
<td>50.2</td>
<td>0.301</td>
</tr>
<tr>
<td>Glucose-1-P</td>
<td>82.5</td>
<td>85.0</td>
<td>82.0</td>
<td>83.5</td>
<td>0.0783</td>
</tr>
<tr>
<td>Glucose + Glucose-1-P</td>
<td>71.0</td>
<td>66.5</td>
<td>75.0</td>
<td>70.8</td>
<td>0.1503</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>28.0</td>
<td>15.0</td>
<td>15.0</td>
<td>19.3</td>
<td>0.716</td>
</tr>
<tr>
<td>D58 without carbohydrate</td>
<td>90.5</td>
<td>86.5</td>
<td>90.5</td>
<td>89.2</td>
<td>0.0505</td>
</tr>
</tbody>
</table>

To find a common system, or mechanism, involved for glucose and fructose utilization, after an adaptive, or "inductive", phase had occurred during growth in either of the two sugars. Therefore if that were the case, a similarity in growth response would be seen in both glucose and fructose broths, when inoculated with either a glucose- or fructose-grown log phase mother culture since, theoretically, the adaptive (or inductive) state would be most active in cells taken from the exponential phase of growth. To test this, an experiment was carried out employing a glucose-grown, middle log phase, culture as the inoculum. As the results of such an experiment it may be seen
Table 9. Data from an experiment designed to determine whether a combination of α-D-glucose-1-phosphate and glucose would reduce the extended lag phase normally exhibited for the growth of C. thermocellum (157) in glucose-containing broth. The results are for Hungate's modified medium, and are interpreted as the mean of the per cent transmittance and optical density of three culture tubes within each of the five treatments.

<table>
<thead>
<tr>
<th></th>
<th>%T_1</th>
<th>%T_2</th>
<th>%T_3</th>
<th>x %T</th>
<th>x O.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>59.0</td>
<td>57.0</td>
<td>57.5</td>
<td>57.83</td>
<td>0.2384</td>
</tr>
<tr>
<td>Glucose-1-P</td>
<td>85.0</td>
<td>85.0</td>
<td>85.5</td>
<td>85.16</td>
<td>0.0706</td>
</tr>
<tr>
<td>Glucose + Glucose-1-P</td>
<td>76.0</td>
<td>78.5</td>
<td>78.5</td>
<td>77.66</td>
<td>0.1107</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>12.5</td>
<td>12.25</td>
<td>12.25</td>
<td>12.33</td>
<td>0.912</td>
</tr>
<tr>
<td>D58 without carbohydrate</td>
<td>84.0</td>
<td>85.5</td>
<td>86.0</td>
<td>85.18</td>
<td>0.0706</td>
</tr>
</tbody>
</table>

that a distinct preference for cellobiose is retained (Figure 21). The length of the lag phase required for initiation of growth in glucose broth has been considerably shortened, however, and not surprisingly so. But most interestingly, the fructose-grown culture behaved as it normally would, were the inoculum a cellobiose-grown mother culture.

Results with first passage, fructose grown, log phase mother culture employed as inoculum

The preceding experiment was repeated using a fructose-grown, log phase mother culture as the inoculum. The results
Figure 21. Growth response of *C. thermocellum* (157) to cellobiose, glucose and fructose when employing a first passage, glucose-grown, log phase mother culture as the inoculum. The growth medium was Hungate's modified formula.
• BASAL MEDIUM (B.M.) WITHOUT CARBOHYDRATE
△ B.M. + GLUCOSE
○ B.M. + FRUCTOSE
× B.M. + CELLOBIOSE

O.D. AT 340 mμ

0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0

0 10 20 30 40 50 60 70
HOURS
of this experiment (Figure 22) showed that (1) a preference for cellobiose is retained; that (2) a reduction in the lag phase time for growth on fructose is effected; and that (3) growth in glucose broth is similar to the growth patterns observed when the inoculum was a cellobiose-grown mother culture.

The results of the two foregoing experiments would indicate that the systems involved in glucose and fructose utilization were different. If so, then the fourth hypothesis would be incorrect unless, however, two separate extracellular enzymes were involved for the formation of cellobiose: one specific for the catalysis of glucose molecules, produced only by glucose-grown cells; and the other, specific for fructose molecules, produced only by fructose-grown cells. If that were the case, it should be possible to detect at least traces of cellobiose, or perhaps cellodextrins, in filtrates of glucose or fructose-containing growth medium.

**Chromatographic Analyses for Cellobiose, or Cellodextrins, in Glucose and Fructose Broth Culture Filtrates**

Chromatographic analyses were performed on cell-free filtrates of glucose and fructose broth cultures. The broth culture filtrates did not, however, reveal the presence of either cellobiose or any of the larger polymeric units.
Figure 22. Growth response of *C. thermocellum* (157) to cellobiose, glucose and fructose when employing a first passage, fructose-grown, log phase mother culture as the inoculum. Growth medium was Hungate's modified formula.
• BASAL MEDIUM (B.M.) WITHOUT CARBOHYDRATE
• B.M. + GLUCOSE
• B.M. + FRUCTOSE
• B.M. + CELLOBIOSE

O.D. AT 340 m\(\mu\)L

HOURS
Growth Response of \textit{C. thermocellum} to Cellobiose, Glucose and Fructose When Employing Inocula of Different Physiological States-II

The investigator did not find it feasible at this point to perform experiments which would have tested hypothesis \#2 (the remaining of the four hypotheses). Instead, as a useful preliminary approach, two experiments were designed to answer questions which were frequently asked: What would be the resulting fermentative pattern in the several carbohydrate test media if the test cultures were first serially transferred through several passages of either glucose or fructose-containing medium? In other words, how stable are the cultures, with regard to cellobiose preference, after a substantial number of transfers are made in the hexose sugars? These experiments were conducted after five serial transfers were made with the test cultures in broths containing either glucose or fructose as the principal energy source.

Results with fifth-serial-passaged, glucose-grown, mother culture as inoculum

After the sixth serial transfer in glucose broth (Figure 23) a definite preference for cellobiose over glucose is retained, suggesting a stable fermentative pattern for both cellobiose and glucose. The fermentative pattern of the three sugars (Figure 23) in this test is reminiscent of the results obtained after two passages in glucose broth (Figure 21).
Figure 23. Growth response of *C. thermocellum* (157) to cellobiose, glucose and fructose when employing a fifth-serial-passaged, glucose-grown, mother culture as the inoculum. The growth medium was Hungate's modified formula.
- BASAL MEDIUM (B.M.) WITHOUT CARBOHYDRATE
- B.M. + GLUCOSE
- B.M. + FRUCTOSE
- B.M. + CELLOBIOSE

Graph showing the O.D. at 340 mμ over hours.
Results with fifth-serial-passaged, fructose-grown, mother culture as inoculum

The results obtained with the sixth serial transfer, fructose-grown, culture are somewhat different (Figure 24) than with lower-passaged cultures. There is no large difference in time shown for growth initiation between the cellobiose and fructose-grown cells; however, the over-all growth rate and cell density is considerably greater in fructose-grown cultures. It will also be noted that the glucose-grown culture behaved in a manner similar to that observed when the inoculum was of a cellobiose-grown mother culture. The results, which show a preference for fructose over cellobiose at the sixth serial passage, are not fortuitous, since the experiment was repeated on two other occasions, with results which were almost identical.

The observed preference for fructose over cellobiose was interpreted in two ways: The preference for fructose represents a mutational change within the bacterial population, resulting in a selected bacterial population having a preference for fructose over cellobiose. However, if the bacterial population is as stable to genetic change toward fructose as the glucose-grown culture would appear to be for glucose, then another explanation would be in order. The change in preference for fructose over cellobiose might indicate inducible enzyme synthesis of one (Eq. 1) or more of the enzymes of the Myerhof-Embden-Parnas pathway, the result of which may possibly be
Figure 24. Growth response of \textit{C. thermocellum} (157) to cellobiose, glucose and fructose when employing a fifth-serial-passaged, fructose-grown mother culture as the inoculum. Growth medium was Hungate's modified formula.
reflected in a faster growth rate of the organisms. In this connection, it is possible that the enzyme glucokinase (Eq. 2) may also be induced, subsequent to or in concert with, the "inductive permeation" process shown for glucose-grown cells (Table 10). This is suggested since, during studies of cellobiose phosphorylase (Eq. 3) - an intracellular enzyme produced in abundance by *C. thermocellum* - only traces of glucokinase were detected (Sih and McBee, 1955; Alexander, 1968a).

Eq. 1  Fructose + $H_3PO_4$ \(\text{Fructokinase}\) Fructose-6-phosphate

Eq. 2  Glucose + $H_3PO_4$ \(\text{Glucokinase}\) Glucose-6-phosphate

Eq. 3  Cellobiose + $H_3PO_4$ \(\text{phosphorylase}\) Glucose+glucose-1-phosphate

To the knowledge of the author, the phenomenon of inducible enzyme synthesis has not been demonstrated for any of the enzymes of the Myerhof-Embden-Parnas pathway. That would not, however, preclude the possibility of this existing in *C. thermocellum*. 
Evidence for Inductive Permeation as an Explanation for the Extended Lag Phase Shown of Cultures Grown in Glucose Broth

Permeation studies with $^{14}$C-glucose

Finally, experiments were conducted that would test hypothesis #2, which stated that cells must undergo some physiological adjustment, or transport induction, before glucose or fructose are taken up by the cell. As has been shown, a log phase, cellobiose-grown, mother culture exhibited a marked lag phase when inoculated into a glucose or fructose-containing medium. This behavior might indicate a temporary lack of permeability for hexoses. Theoretically, then, if this were the case, one should be able to find glucose permeable cells as the culture enters into the logarithmic phase of growth, when cells are grown in broth containing glucose as the principal energy source. This would appear to be the case since, when glucose-grown cells, harvested from the logarithmic phase of growth were exposed to $^{14}$C-glucose, the radioactivity of these cells was considerably higher than cellobiose-grown cells treated in the same manner (Table 10). It is this investigator's impression, therefore, that these data would suggest the existence either of an inductive permeation effect, or enhanced binding and uptake of labeled glucose by glucose-grown cells, and hence would explain the extended lag phase shown by cells grown in glucose-containing broth.
Table 10. Differential affinity for C<sup>14</sup>-glucose of log phase, glucose-grown cells compared with log phase, celllobiose-grown cells

<table>
<thead>
<tr>
<th>C&lt;sup&gt;14&lt;/sup&gt;-glucose exposed</th>
<th>Expt. no. 1</th>
<th>Expt. no. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-grown</td>
<td>770.20</td>
<td>967.96</td>
</tr>
<tr>
<td>Celllobiose-grown</td>
<td>80.40</td>
<td>130.65</td>
</tr>
<tr>
<td>Cells without carbohydrate</td>
<td>48.00</td>
<td>90.00</td>
</tr>
</tbody>
</table>

<sup>a</sup>1 ml sample conc.: 0.3 O.D. units, or 50% transmittance.

Permeation studies with C<sup>14</sup>-fructose

It was then speculated that the lag phase shown for cells grown in fructose broth also might be explained on the basis of permeability, as indicated in the case of the glucose-grown cells. Unfortunately, the results of the one experiment conducted provided no evidence for C<sup>14</sup>-fructose uptake. A repeat of this experiment was attempted on two other occasions, but due to autolysis of the cells occurring during the pre-labeling, preparatory washing stage, the experiments could not be completed.

On the basis of only this one completed experiment, it seems premature to speculate on the possibility that a different mechanism than that seen for glucose uptake was involved for the uptake of fructose in fructose-grown cells. What is more probable (if the one experiment was accurate) is that the
difference in results obtained is due, not to a difference in the mechanism of carbohydrate uptake, but rather to a difference in the experimental conditions needed for demonstrating this same effect.

Perhaps apropos of the latter discussion on permeability is a report by Hoffee et al. (1964) which provided evidence that the glucose transport system in *Salmonella typhimurium* and *Escherichia coli* is unique in possessing an energy-requiring exit reaction which is apparently absent from other permease systems so far characterized.

**Behavior of Two New Cellulolytic Clostridial Isolates Toward Cellobiose, Glucose and Fructose**

In addition to the three strains of *C. thermocellum* thus far discussed, two additional pure cultures of thermophilic, cellulose-digesting clostridia were isolated. The cultures, designated LQ-1 and LQ-8, were isolated from barnyard soil and fresh rumen ingesta, respectively. These cultures were only partially characterized, but from present taxonomic information the organisms could be only one of two species, namely, *C. thermocellum* or *C. thermocellulaseum* (Bergey's 7th ed.). The two cultures behaved toward cellobiose, glucose and fructose in a manner similar to that of *C. thermocellum* strains TET, 157 & 651. These cultures also exhibited preference for cellobiose over glucose and fructose, but only one of the two cultures (LQ-1) appeared to ferment fructose.
It should be mentioned that the two cultures (LQ-1 & LQ-8) were grown in Hungate's modified medium, and although they fermented glucose and fructose in this medium, autolysis was frequently encountered in the early log phase period of growth, when glucose or fructose were employed as the carbohydrate source; however, autolysis was not observed in these cultures when cellobiose was employed as the principal energy source. The same type of lytic behavior was shown by \textit{C. thermocellum} strain 651 when it was grown in Hungate's modified medium containing glucose as the carbohydrate source, but the lytic activity was corrected when this culture was transferred to D58 medium. Perhaps the autolysis is a consequence of some deficiency in the basal growth medium. In this context, it may be noteworthy that a similar autolytic phenomenon was observed in two thermophilic strains of \textit{Bacillus coagulans} (Marshall and Beers, 1967). In those cultures the autolytic problem was corrected by the addition of adenine to the growth medium.

A possible connection between autolysis and temperature relationship of the organism ought not to be overlooked. Although it has been reported that the optimal temperature for \textit{C. thermocellum} is between 55-65°C, most of the work reported for the pure culture was conducted at 55 (McBee, 1948, 1950; Sih \textit{et al.}, 1957). Other investigators (Alexander, 1961, 1968a, b; Sheth and Alexander, 1967, 1969), working with \textit{C. thermocellum} strain 651, conducted all of their enzyme studies with cells grown between 50-55°C. Studies in this laboratory with
Taxonomic Considerations of Clostridium thermocellum

In 1954, Enebo reported on an anaerobic, thermophilic, cellulolytic culture which he named Clostridium thermocellulaseum (Bergey's 7th ed.). This organism appeared to be very similar to C. thermocellum (Bergey's 7th ed.), the main difference being that C. thermocellulaseum fermented glucose, fructose and arabinose, whereas C. thermocellum did not. Bergey's Manual (7th ed.) listed an additional difference between the two species by stating that mannose and maltose are "weakly" fermented by C. thermocellulaseum. However Enebo (1954), in describing this organism, reported that "mannose and maltose are fermented only sporadically." He apparently considered this characteristic to be dubious since, in comparing the two organisms, he chose not to include mannose and maltose as characteristics which served to distinguish the two species.

In view of the findings in this investigation that C. thermocellum does ferment both glucose and fructose, it seems hardly appropriate to consider C. thermocellum and C. thermocellulaseum as two separate species, since the only remaining significant difference between the two species is a matter of difference in fermentative pattern on one carbohydrate. The thought of resolving this difficulty by base-ratio comparisons of the two species was considered. Unfortunately, the culture
of *C. thermocellulaseum* is not available in the stock culture collection of ATCC, and a request for a culture of this organism addressed to Dr. L. Enebo was unanswered.
SUMMARY

The underlying reason(s) for the inability of *Clostridium thermocellum* to utilize glucose on Hungate's standard or basal medium was investigated. It was found that with an increased concentration (0.45%) of yeast extract added to this growth medium, both glucose and fructose were fermented by *C. thermocellum* strains TET and 157; and although *C. thermocellum* strain 651 fermented glucose, it did not appear to ferment fructose even with this increased level of yeast extract.

The stimulatory effect of heated carbohydrates on the growth rate of *C. thermocellum* was briefly investigated. It was observed that for both glucose and cellobiose, sugars heated in absence of Hungate's basal growth medium were less stimulatory than those heated together with this basal growth medium, but were more stimulatory than were filter-sterilized sugars.

Growth factors such as asparagine, glutamine and biotin were tested as possible stimulatory agents involved in glucose fermentation by *C. thermocellum*. These compounds failed to stimulate glucose fermentation, as evidenced by the inability of the organisms to grow in basal medium supplemented with these agents. Only one concentration of these compounds was tested: 23 mg% for asparagine; 30 mg% for glutamine; and 16 μg/L for biotin. In retrospect, however, perhaps higher concentrations should have been employed.
The basis for the extended lag phase shown for cultures grown in glucose and fructose broth was investigated. It was found that log phase, glucose-grown, cells had an affinity for $^{14}$C-glucose, whereas cellobiose-grown cells treated in like manner did not. This was interpreted as meaning that the delayed lag phase shown for cells grown in glucose-containing broth was due to an inductive permeation effect by glucose. An experiment directed at an explanation for the delayed growth in fructose broth was conducted by exposing log phase, fructose-grown cells to $^{14}$C-fructose. Unlike the experiments with glucose-grown cells, these cells did not show any $^{14}$C-fructose uptake. However, on the basis of one experiment it would appear illogical to conclude that the mechanism for glucose and fructose uptake were different. What is more probable is that the experimental conditions needed to effect the same results are different—assuming the accuracy of the one experiment conducted with fructose.

Two new anaerobic, cellulolytic, thermophilic isolates showed a behavioral pattern toward cellobiose, glucose and fructose similar to that observed for the three strains of $C.\text{thermocellum}$. It is speculated that, as in this investigation, given the proper growth conditions, all anaerobic, cellulolytic, thermophiles will utilize glucose, since glucose appears to be a normal reaction product of cellulose digestion by all such organisms heretofore studied. Why cellobiose should be preferred to glucose is not known. Perhaps it is due to an
evolutionary selection of an organism which is more adapted to the fermentation of cellobiose, because of the competition for glucose by the microorganisms of the surrounding eco-system, together with the fact that relatively few microorganisms are capable of fermenting cellobiose.

In view of the findings in this investigation that C. thermocellum ferments glucose and fructose; and since the only remaining significant difference between C. thermocellum and Clostridium thermocellulaseum is a fermentative pattern involving one carbohydrate, arabinose, it is felt that the two organisms should be considered to be members of the same species.
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ACKNOWLEDGEMENTS

The author wishes to express his appreciation to Dr. L. Y. Quinn, his major professor, for his kind, generous, and unreserved assistance throughout the course of this investigation; and also for his competent guidance in academic affairs.

Appreciation is extended to members of the author's graduate committee for their help and suggestions when these were needed, and for their vote of confidence in having allowed him to pursue this advanced academic training.

Thanks are also due to other members of the Bacteriology Department, both staff and fellow students, for their helpful suggestions which contributed considerably to a successful completion of this project.