AN ABSTRACT OF THE THESIS OF

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Title: THE INFRA-RED ABSORPTION SPECTRA OF THE CLOSTRIDIA

Abstract approved: Redacted for Privacy
K. S. Pilcher

There are six types of Cl. botulinum (Types A, B, C, D, E, and F). The differentiation between these types is based upon the antigenic structure of the toxin. Many strains of these various types have been isolated. In addition, a number of nontoxigenic "type E-like" strains have come to light. It would be of great value to have a rapid method of distinguishing between these strains, or of identifying the type of toxigenic culture. This thesis discusses an investigation into the possibilities of the use of infra-red in an approach to this problem. Smears of bacterial cells were prepared by scraping a small amount of growth from the agar surface of a petri plate, and dissolving it in a drop of water on the surface of a silver chloride disc. In the case of broth cultures, growth to be placed on the discs was first obtained by centrifugation. A second empty disc was then placed over the first with a bit of lens tissue under one edge to prevent the formation of interference fringes. The two discs were

then taped together and placed in the IR spectrophotometer, together with a pair of reference discs for the recording of the IR spectra of the whole bacterial cells.

1. Preliminary work indicated that the most reproducible section of the IR spectrum, which would also give unique and somewhat characteristic variation was to be found in the region from 7.0 μ to 10.0 μ.

2. It was further found that the characteristic variations of an organism were magnified to the greatest extent when the smear was of such a thickness that the 7.0 μ to 10.0 μ region of the spectrum had its entire transmittance lying in the range from 20% to 70%. The preparation of smears of the desired thickness became somewhat of an art.

3. The great bulk of the clostridia examined fell into four basic spectral patterns based upon variations within this range. The spectral patterns of the clostridia seemed to be unique from that of several other bacteria which were examined.

4. The age of the culture has a rather profound effect on the IR spectrum of a culture. The variability of the IR spectrum of a culture decreases with age, between two and five days. One pattern, which is seen to predominate at the two day age, completely disappears at the five day age. The changes were seen to be associated with the accumulation of small amounts of glycogen within the cells as the culture ages. These changes seem to be especially associated

with Cl. botulinum type E.

5. It is concluded that the technique has little value as an aid in the identification of clostridial species. Most of the pathogenic clostridia could not be distinguished from the non pathogens by this means. Neither could the toxigenic strains of Cl. botulinum type E be distinguished from the non toxigenic "E-like" strains. The technique may have some value in monitoring physiological changes within a given culture or group of cultures, however.

6. Washing the cells before preparing the smears seems to decrease the intensity of the region of the spectrum from 6.2 μ to 8.2 μ. This is the region associated with the types of bonds found in proteins. Changes after washing also occur in the region of the IR spectrum from 8.5 μ to 10.5 μ. This region is associated with bonds found in nucleic acids and polysaccharides.

7. The IR spectra of purified and unpurified spore preparations indicates that the spectra of the spore may vary considerably from the vegetative cell, but no distinctly new types of spectra are seen.

The Infra-Red Absorption Spectra of the Clostridia

by

Donald Kurt Pfeifer

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INTRODUCTION

The drama and suddenness with which an outbreak of botulism may occur, have made this subject a matter of continuing concern in recent years. According to Doleman and Murakami (1961), the causative organism, Clostridium botulinum, may be subdivided into six types. This subdivision is based upon the antigenic structure of the toxin, and the various types have been labeled A, B, C, D, E, and F.[1] Osheroff, Slocum, and Decker (1964) state that most occurrences in the past three or four decades were associated with home canned foods. The responsible organisms were types A and B. Type E has, however, been the most prevalent cause of outbreaks from commercially prepared foods in recent years (especially fish).

Many strains of these six types have been isolated. Dolman (1957) states that about 100 type E cultures alone have been isolated. In addition, a number of antigenically and physiologically similar, but nontoxigenic strains have come to light. He further says that it is quite common for the toxigenic strains to revert to a nontoxigenic state. The mechanism of this reversion is not known yet.

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[1] Types A and B were first described by Burke (1919), type C was described by Bengston (1922), type D was described by Meyer and Gunnison (1929), type E was described by Gunnison, Cummings, and Meyer (1936), and type F was described by Dolman, C. E. and Murakami, Lily (1961).

It would be of great value to have a rapid method of distinguishing between these strains, or of identifying the type of a toxigenic culture. This thesis discusses an investigation into the possibilities of the use of infra-red in an approach to this problem. A cross section of the infra-red spectra of other important clostridia is also considered.

Infra-red spectrophotometry has long been of value to the chemist. The infra-red absorption spectrum of a molecule is a summation of the vibrational and rotational motions of all of the atoms within it. It is found that the various structural groups of a large organic molecule each have unique absorption bands. The sum of all of these bands represents the total spectrum of the molecule. These bands may be large or small, simple or complex. Each type of molecule, except optical enantiomorphs, has its own spectrum or "fingerprint."

Since bacteria may be compared to "living bags of these large molecules," it is logical to consider that each "bag" is a unique combination of them. Being unique, each bacterium should then have its own spectral pattern. Unfortunately this simple picture is complicated by several factors. When a large mixture of these large organic molecules are examined by means of infra-red, their unique absorption patterns tend to merge into diffuse regions of maxima and minima. Also, many of the molecules which make a cell unique are present in such small quantities that they are literally drowned out by the

absorption of the major constituents of the cell, the proteins, carbohydrates, and nucleic acids. These three classes of compounds, have very distinct and unique peaks. All proteins have very similar spectra, since all are composed of large numbers of carboxyl and amino groups. Carbohydrates and nucleic acids each have their own unique absorption regions, but the difference between any two carbohydrates or any two nucleic acids is very small.

Another complicating factor is that water, which is present in large quantities in all actively living organisms, has very strong absorption bands at about 3 and 6 μ. This can be partially compensated for by thoroughly drying the organism in an evacuated desiccator over P_2O_5, or by recording the spectra first in water and then in heavy water (heavy water absorbs at 4 and 8 μ). In this study, the organisms were placed on silver chloride discs and dried as described above.

Biological materials are generally examined in the region from 1-16 μ. Norris (1959, p. 327) says,

> Absorption bands between 2 and 7 μ can usually be associated with the fundamental vibration of a particular group or bond in the molecule. For instance, the stretching vibration of a nitrogen-hydrogen bond absorbs radiation of wavelength 3 μ, and the stretching vibration of a carbon-oxygen double bond absorbs near 6 μ. At wavelengths greater than 7 μ, absorption bands arise usually from skeletal vibrations of the molecule and can rarely be associated with the presence of a particular group of atoms. Absorption bands between 7 and 15 μ tend to be characteristic of the whole molecule and have often been likened to a molecular fingerprint.

Although the spectra of different bacteria are very similar, there are slight inflections in regions of absorption maxima and minima which may be unique to different types of bacteria. Occasionally a compound which is present in cells in very large quantity, may show up on the spectral pattern very distinctly; however, great caution must be used when inferences about the chemical constituents of the cell are made from spectra of the whole cells. Many workers try to use these slight differences as an empirical means of distinguishing between strains.

Figure 1 illustrates the absorptions of the various structural groups most commonly found within bacterial cells. These absorption regions are superimposed on a typical bacterial spectrum to illustrate the probable significance of the main absorption bands of the bacterial cell.

Figure 1. The typical absorption spectrum of whole bacterial cells, illustrating the absorptions of various chemical bonds.

A. N-H, O-H Stretching

B. C-H

C. C=O Stretching, C=C, C=N

D. CONH, N-H

E. P=O

F. Region from 8.5 μ to 10.5 μ, C-C-C vibrational modes, C-O stretching vibrations, O-H deformations (Norris, 1959).

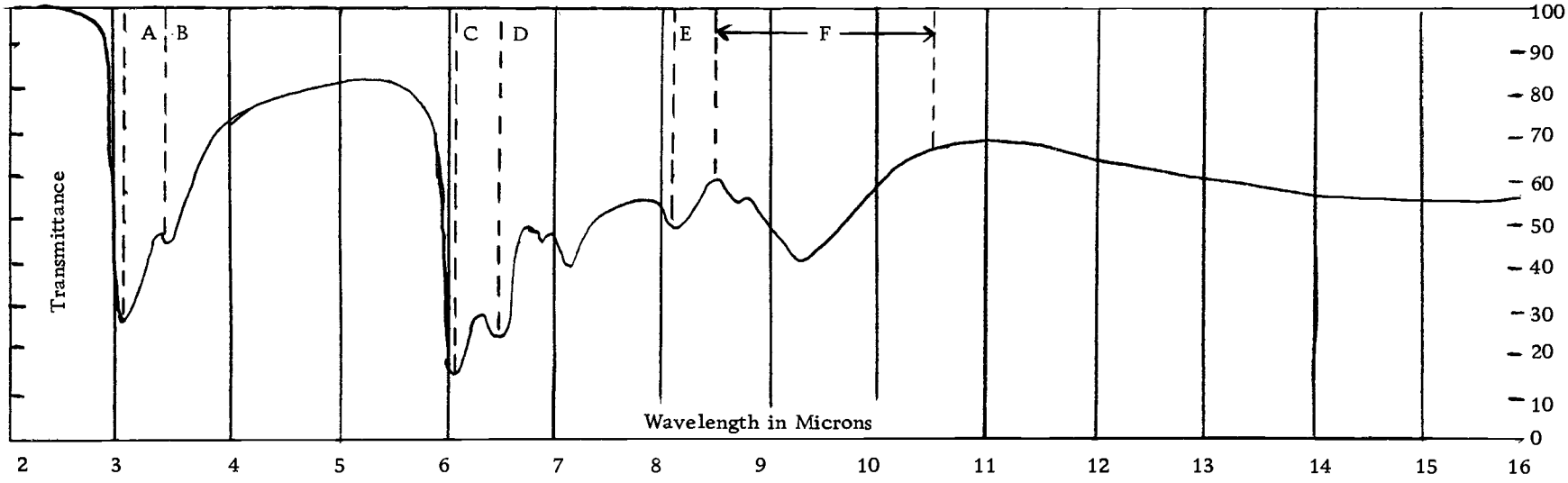
A
B
C
D
E
F
Transmittance
Wavelength in Microns
100
90
80
70
60
50
40
30
20
10
0
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16

Figure 1.

HISTORICAL REVIEW

One of the earliest attempts to record the infra-red spectra of biological materials was by Stair and Coblentz (1935). They examined a wide range of materials such as onion skins, egg membranes, gelatin, and bat's wings. The similarity of these spectra was noted. It is now known (Norris, 1959) that the spectra of all tissues is quite similar.

Randall et al. (1951) published a paper describing the use of infra-red in the differentiation of strains of mycobacterium. In a series of later papers, he described the differentiation of these organisms by the spectra of their extracted and fractionated lipids.

Stevenson and Bolduan (1952) described a technique for obtaining the spectra of dried bacterial films on silver chloride discs.

It was concluded that the differences in the absorption spectra of the organisms in the study were sufficient and consistent enough to differentiate the species studied. The groupings by infra-red spectrophotometry did not always follow the groupings as defined in Bergey's Manual. It was finally concluded that the use of infra-red spectrophotometry in the classification and differentiation of bacteria seemed promising. Levine et al. (1953b) cites evidence to indicate the 6.05 and 6.45 μ bands, in spectra of bacterial cells, are derived from the peptide linkages of proteins, that the 8.0 to

8.1 μ band is due to nucleic acids, and that the intense absorption band from 8.6 to 10.0 μ is due to a combination of nucleic acids and carbohydrates. When these three components are extracted from cells separately, the absorption bands attributed to them disappear. It was felt, by this group, that the use of infra-red spectra in the differentiation of microorganisms held great potential, but since the absolute value of the IR spectrum is dependent on many complex variables, only a consideration of the general shape and relative depth of the bands could be made.

It was noted (Levine, Stevenson, and Bordner, 1953a) that when cells are grown in a medium containing glucose or other usable sugar, absorption bands at 8.7, 9.25, and 9.75 μ were seen. When the carbohydrate was not added to the medium, only the 9.25 μ band was seen. These additional absorption bands could be just weak inflections, or very strong. Rupture of these cells by sonication, followed by centrifugation, extraction with ethanol, and removal of protein by shaking with chloroform and isoamyl alcohol produced a product with an infra-red spectrum identical to a commercial glycogen preparation. When iodine was added to the product, a reddish brown coloration was noted. This is typical of glycogen.

This topic is further discussed in another paper (Levine et al., 1953c). It is stated that glycogen has weaker bands at 10.75, 11.8, 13.2, and 14.2 μ, but the bands at 8.7 and 9.75 μ are of especial

value, because of the absence of bands due to other interfering cellular components at these wavelengths. Since the peaks at these points are also dependent on the thickness of the smear, an internal reference must be made to correct this. This reference is the absorption at 6.45 to 6.50 μ. A ratio of the absorption at 9.75 μ to the absorption at 6.5 μ was plotted against a standard chemical glycogen analysis to form a reference curve for the determination of the percentage of glycogen within the cells of a dried smear.

A marked variation in production of this glycogen-like component was seen between various strains of the same organism, but this is complicated by the fact that it may show up late and disappear early. A temperature of 15°C to 20°C seemed to be more conducive to production of this substance than a temperature of 37°C. Its appearance and disappearance was also slower at this temperature, however. Various sugars were tried, but all seemed to produce this effect. A sugar concentration of one percent resulted in maximal production. Production of this substance was inhibited by concentrations of 0.0002 percent sodium azide, and 0.0013 percent sodium iodoacetate.

The results cannot be rigorously quantitative, because the dried films are not completely homogeneous. Nonhomogeneous systems do not follow Beer's law (Jones, 1952). Levine et al. (1953c) further notes the dangers of attempting to correct for the absorption

of the silver chloride disc by placing an additional one in the reference beam of the spectrophotometer, since the absorption and reflections of the two discs may not be equal. Capsules or slime may interfere with or hide the presence of the glycogen-like substance.

Blackwood and Agnes (1957) noticed a unique absorption peak at 5.7 μ, while examining various strains of the genus Bacillus, grown on media containing a fermentable carbohydrate. Only those cells containing numerous large fat globules exhibited this phenomenon. This peak was subsequently associated with polymerized β hydroxybutyric acid. It was mentioned that this was the only noticeable difference that could be seen between the strains.

Shortly thereafter, Haynes et al. (1958) published a report which filled in information as to the cultural factors affecting the production of this substance. Genus Bacillus seemed to be unique in exhibiting these properties. All of the strains of Bacillus cereus examined exhibited this property, but only certain strains of Bacillus megaterium were implicated.

The presence of 1% glucose in the medium seemed to be a necessary prerequisite to the formation of the 5.7 μ band. When NH_4NO_3 was the principal nitrogen source, a better contrasted curve seemed to result. It was found that galactose, glycerol, and fructose were often just as effective as glucose in producing the 5.7 μ band. The time of appearance and disappearance of this band seemed to vary

with the strain investigated. Correlated with the intense 5.7 μ band were two other small bands at 7.6 μ and 8.5 μ. The substance producing these bands was subsequently isolated and shown to have a spectrum identical to that of polymerized β hydroxybutyric acid.

Between 1950 and 1958, a large number of organisms and their products were examined by means of infra-red spectroscopy in an attempt to establish a scheme of classification. Among these were Levine et al. (1954), who typed Klebsiella, and Benedict (1955), who classified virus preparations.

A paper was published by Kenner (1958), describing the effect of instrumental and environmental variables on the spectra of bacteria. It was shown that a large difference between 8.4 and 12.0 μ occurred in the spectrum of cultures of Serratia marcescens incubated at different temperatures. Some variation in the spectra of cultures harvested at different ages was noted. As shown above, the presence of fermentable carbohydrates in the media can cause marked variation in the spectrum. The presence of bile salts can cause problems, because some of the salts may be precipitated and harvested with the bacteria to cause bizarre alterations in the spectra. The culture media must be removed from the cells, since it can also alter the spectrum.

It was reported that pathogens could be easily studied by first autoclaving them. Since this produced somewhat of an alteration of

the spectra, an unknown organism would have to be compared with the known spectra of other autoclaved specimens.

It is claimed that when these sources of error are controlled, reproducibility within 2% may be had in the range from 5.6 to 12.0 μ.

Riddle (1956), says that the differences between the spectra of microorganisms lies primarily in the percentages of absorption at different wavelengths, rather than the presence of differing absorption bands.

The development of a system for recording the infra-red spectra of bacteria on IBM cards was reported. This enabled a catalogue of known spectra to be built up, against which the spectrum of an unknown organism could be compared. The IBM card also contained coded information as to the morphology, gram reaction, and culture media used. It is admitted that a number of organisms had completely similar spectra, and could not be distinguished by this method. On the other hand, some organisms in the genus Bacillus produced such a high degree of variability that comparisons could not be made very well.

O'Connor, McCall, and Dupre (1957) examined the infra-red spectra of the acetone extracts of bacteria. Since most of the classification attempts reported in the literature have been based upon very slight inflections in the spectral curves of different organisms, the reported differences were hardly above that which would be expected

by chance variation alone. It was thought that any actual differences which would show up on the spectrum of the entire cell would be representative of compounds present in minute quantity within the cell. It would then follow that if these compounds could somehow be extracted from the rest of the cellular components, and the spectra of these compounds alone could be examined, the differences would be greatly magnified. Various organic solvents, it was postulated, could be used to extract these components after rupture of the cell by some means. A number of such solvents were tried, and acetone was selected because it produced the best spectrum.

Examples are shown in the paper to illustrate that differences will show up between the acetone extracts of organisms when no differences can be seen between the smears of whole cells. The suggestion is made that if the acetone extractions by themselves do not indicate differences, repeated extractions with a series of other solvents could be made until a satisfactory one is discovered.

As time passed, warnings (as to over optimistic and irrational analysis of minute inflections in the infra-red spectra of micro organisms) began to be issued. Kull (1956, p. 106) states,

> Although the IR spectra of purified extracts or of relatively simple components of closely related species may be used as a criterion for separation or distinguishing studies, the application of IR as a qualitative differentiating tool is limited if crude extracts, whole or intact cells are used. Positive identification of unknowns solely by the IR method can not be made.

Despite this warning, papers continued to be published, supposedly differentiating bacteria on the basis of their infra-red spectra. These publications ceased to appear in American journals to a large extent, by the end of the fifties; however, reports in foreign journals continued during the sixties.

The warning was again repeated by Wright and Lockhart (1965). They say that the variation in the spectral pattern of one organism in a defined medium, with different limiting substrates, shows more variation than those reported as significant in many of the taxonomic studies. It is suggested that the use of infra-red may be of far more value in extended physiological studies of one organism under varying growth conditions than in taxonomic studies. The differentiation of species within a genus has been often based on differences smaller than those found in a single strain of bacteria due to chance, or a small change in the environment.

Smith et al. (1957) concludes that there is little value in the use of infra-red in taxonomy, when it must compete with techniques such as fluorescent antibodies.

MATERIALS

Organisms Used

Clostridium botulinum Cultures

Type A	Type B	Type F
No. 5A	No. 113B	OSU-Craig
No. 33A	No. 115B	

Type E

Toxigenic cultures	Nontoxigenic "E-like" cultures
066B	34-1
VH#1	805Bb
VH#2	170C
070	066BNT
Saratoga	170IX
Beluga	15CbTi
Iwahai	15Ati
Isolate #1	S-9
Isolate #2	GB-3
Isolate #3	8ATi
Kalamazoo	900D

Detroit

Tuna Can

Alaska

Miscellaneous Clostridia

Cl. welchii - OSU collection

Cl. novyi - OSU collection

Cl. sporogenes - OSU collection

Cl. histolyticum - OSU collection

Cl. fallax ATCC #19400

Cl. tertium #19405

Cl. difficile #9689

Cl. tetani #9441

Cl. butyricum #19398

Miscellaneous Bacteria

Bacillus subtilis

Bacillus megatherium

Sarcina lutea

Staphlococcus aurens

Media Used

Cooked Liver Medium

Beef liver	500.0 gm.
Tap water	1000.0 ml.
Peptone	10.0 gm.
Dipotassium phosphate (Anhydrous)	1.0 gm.
Final pH	7.0

The beef liver was cut into very small chunks and soaked in one liter of water over night in the refrigerator. After the fat was skimmed off the top, the mixture was heated in the autoclave for 10 min. at 121°C. Then the meat was removed from the broth by filtering through cheese cloth, and saved. Peptone and phosphate were added to the broth, and it was heated to 100°. The reaction of the medium was adjusted to pH 8.4, it was filtered through paper, and made up to one liter with more water. A small amount of powdered $CaCO_3$ was added to each test tube before adding about one half inch of beef liver chunks. It was covered with broth to a depth of about two inches, and sterilized for 15 minutes at 15 lbs. pressure.

Immediately before inoculation, tubes of the medium were heated in a boiling water bath and cooled quickly. After inoculation, the culture was sealed with vaspar to insure anaerobic conditions.

This medium was used to store the stock cultures.

TPGY Medium

Trypticase	50 gms.
Peptone-Difco	5 gms.
Glucose-Baker (Reagent)	4 gms.
Sodium thioglycollate-Difco	2 gms.
Yeast Extract-Difco	20 gms.
Agar (if desired)	15 gms.
Distilled water	1000 ml.
Final pH	7.0

All of the dry ingredients except the agar were mixed. Water was added, and it was stirred until completely dissolved. The pH was adjusted to 7.0, then agar was added, and it was heated in a steamer until the agar was completely dissolved. It was dispensed into screw capped tubes (15 ml. per tube) and autoclaved at 15 psi. for 15 minutes. The pressure was allowed to come down slowly.

TPG Medium for Biphasic Cultures
(Schmidt, Lechowich and Folinazzo, 1962)

Trypticase	50 gms.
Peptone	5 gms.
Glucose	4 gms.
Sodium thioglycollate	2 gms.

Water	1000 ml.
Agar	30 gm.
Final pH	7.0

The dry ingredients were mixed together well. Water was added and it was stirred until they were dissolved. The pH was adjusted to 7.0, then agar was added and it was heated in a steamer until it was completely dissolved. It was autoclaved at 15 psi. for 15 minutes. The pressure was then allowed to come down slowly.

Anaerobic Indicator (Davidsohn and Wells, p. 934)

Solution A:

N/10 NaOH, 6 ml
Add distilled water to 100 ml

Solution B:

0.5% Methylene Blue, 3 ml
Add distilled water to 100 ml

Solution C:

6 gm. glucose
Add distilled water to 100 ml

Place equal volumes of each solution in a test tube. Add one small crystal of thymol. Boil until colorless. Place in an anaerobic jar. Remains colorless if anaerobic conditions are maintained.

Infra-red Spectrophotometer

Beckman Model IR-5 and stainless steel window holders #N-05, Chart Paper #18920.

Beckman Instruments Inc. 2500 Fullerton Rd., Fullerton, California.

Silver Chloride Windows

#02505, Harshaw Co., 2235 E. Middlefield Rd., Mountain View, California 94040.

Sonicator

Branson Sonifier Cell Disruptor, Model #W-140C

Serial NOS. C7070 TO Heat Systems Co., 60 Broad Hollow Road, Melville, N. Y. 11746.

Water Bath

Cat # 13700 Serial #0865

Chicago Surgical and Electrical Co., Melrose Park, Ill.

Acetone

Reagent A. C. S. Code 1004, Allied Chemical Co., General Chemical Division, Morristown, N. J., USA.

Glass Beads

Glasperlen, Kat. Nr. 54140 (2883), 0.11-0.12 Dia.

B. Braun Apparatebau, Melsungen

Made for Bronwill Scientific Inc., Rochester, N. Y.

Alumina

Chromatographic grade (80-200 Mesh) #9296 AX612

Matheson, Coleman, and Bell, Norwood (Cincinnati), Ohio,

and East Rutherford, N. J.

Carbowax

Polyethylene Glycol 4000, Ave. Mol Wt. 3000-3700.

Baker Grade #U221, J. T. Baker Chemical Co., Phillipsburg,

New Jersey.

Nitrogen

96% Nitrogen, 4% Carbon Dioxide (Dry, High Purity)

National Cylinder Gas, Div. of Chemetron Corp, Chicago, Ill.

Centrifuge

International Centrifuge Size 2 Model V No 9443A, Volts 113,

cy 50-60 AMPS 6, 3/4 H. P. International Equipment, Co.

Boston, Mass.

Microscope and Camera

E. Leitz (GMBH) Wetzlar (Germany)

Nr. 501778 U. S. Distributors, E. Leitz Inc.

468 Fourth Ave. New York 16, N. Y.

METHODS

Routine Methods

Petri plates of TPGY agar were inoculated by spreading 0.3 ml. of a 24 hr TPGY broth culture of the organism to be used, evenly over the surface of the plate with a sterile, bent, glass rod. The plates were then placed, upside down, into a Case Jar along with an anaerobic indicator. After evacuation and refilling of the jar three times with a 96% nitrogen, 4% carbon dioxide mixture, they were placed into a 28°C incubator for either two or five days.[2]

Upon removal from incubation, the organisms were harvested by lightly scraping the surface of the agar with a rubber policeman. A small amount of this growth was then placed on the surface of a silver chloride disc and suspended in a few drops of distilled water by agitation with the rubber policeman. It became necessary to make a thick and a thin suspension on each of two discs respectively, to insure an adequate spectrum from each culture. The discs were then dried in an evacuated desiccator, containing P_2O_5, overnight.

Just prior to IR examination, a clean dry disc was placed over the one containing the specimen, and they were taped together with

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[2]One of the main objectives of this work was to determine if any difference exists between the IR spectra of the toxic and nontoxic strains of Cl. botulinum type E. Since it is known that good toxin production is seen after five days at 28°C, it was necessary to compare the spectra of two day cultures against the five day cultures, and also to compare the spectra of five day toxic strains against five day nontoxic strains, in addition to the two day comparisons.

cellophane tape around the circumference. Interference fringes were prevented by placing a bit of lens tissue (rolled up to about a 2 mm. thickness) between the discs at one side next to the edge. A pair of clean dry discs were used as a balance in the reference side of the spectrophotometer. This same pair of discs was always used in this manner. Before insertion of the specimens, both beams of the spectrophotometer were blocked in a manner to hold the recording pen at 50 percent transmittance. Then the balance knob was adjusted until the pen ceased to drift. A control spectrum of air against air was taken each day.

It was found that the discs could be safely disinfected after use, by immersing in a 0.53% sodium hypochlorite solution for one half hour. This did not seem to affect the absorption of the discs themselves. After disinfection, the discs were thoroughly washed with distilled water and wiped gently with lens tissue.

Because of the large number of strains and species worked with, it would have been too time consuming to completely check the identity of each culture; however, each culture was checked for its Gram stain reaction, presence of spores, and heat resistance. Of all of the cultures worked with, only Cl. botulinum strain 900D, and Cl. fallax failed to pass these tests. These two organisms are not considered in the final analysis of the results.

Broth Cultures

It was thought that nutrients from the agar, and metabolic excretions of the organisms might be contributing to the spectra. To test the effects of this, cultures were grown in TPGY broth. The organisms were harvested by centrifugation at 3000 rpm for ten minutes, then washed twice with distilled water by centrifugation in the same manner, before making spectra.

Spore Cultures

Spore cultures were grown using the biphasic culture technique as described by Bruch and Bohrer (1968). The liquid phase was distilled water, and the solid phase was TPG agar (3% agar). The cultures were incubated at 28°C for two days, then placed in the refrigerator overnight for autolysis to take place (Bruch and Bohrer, 1968). They were next frozen and thawed five times in an acetone dry ice mixture to insure lysis of the vegetative cells. A very high degree of sporulation was attained with Seratoga and Beluga strains of Cl. botulinum type E, and it was thought that satisfactory spore smears could be made from the unpurified cultures. In the case of the other strains however, only one fourth to one half of the cells sporulated, and it became necessary to purify them before making spectra. (See Figure 3.)

The technique of separating the spores from the cells and cell debris used the two phase polyethylene glycol, phosphate buffer system described by Sacks and Alderton (1961). A culture of 100 percent clean free spores was attained in this manner. After washing several times with distilled water, an aqueous suspension of the spores was placed on the silver chloride discs, and dried as described above.

Acetone Extracts

O'Connor, McCall, and Du Pre (1957) reported excellent results in the differentiation of bacteria by infra-red analysis of their acetone extracts. It was decided to repeat this procedure with the Clostridia. They said that rupture of the cells was a necessity to the achievement of satisfactory results.

There are a number of means of rupturing cells. The first procedure attempted was to grind the cells with alumina in a mortar and pestle (McIlwain, 1948). After finding that the abrasive itself contributed to the spectra, the use of alumina was abandoned, and very tiny glass beads were tried (Dockstader and Halvorson, 1950). These also proved to be unsatisfactory, and a final attempt to rupture the cells by means of sonication was made. When this also failed to give reproducible spectra, the entire acetone extract procedure was abandoned.

The Effect of Age on the Absorption of the Silver Chloride Windows

It can be seen (Figure 2) that the silver chloride windows absorb considerably by themselves in the IR region. This absorption increases with age, especially in the lower wavelengths of the spectrum. This could be partially compensated for by placing an additional window in the reference beam, but if the reference window does not have exactly the same absorption as the sample window, variation will still be seen. If the sample window is darker than the reference window, the transmittance will decrease as the wavelength decreases. If, however, the reference is darker than the sample window, the transmittance will increase above 100% in the shorter wavelengths of the spectrum, while it will remain close to the 100% level in the longer wavelengths of the spectrum.

Because of the extreme amount of caution which must be used in interpreting the lower wavelengths of the spectrum, and the lack of anything of interest in that region, the part of the spectrum below 5.5 μ will be omitted in the results given. Also, since the region above 14 μ shows nothing interesting, this will also be omitted.

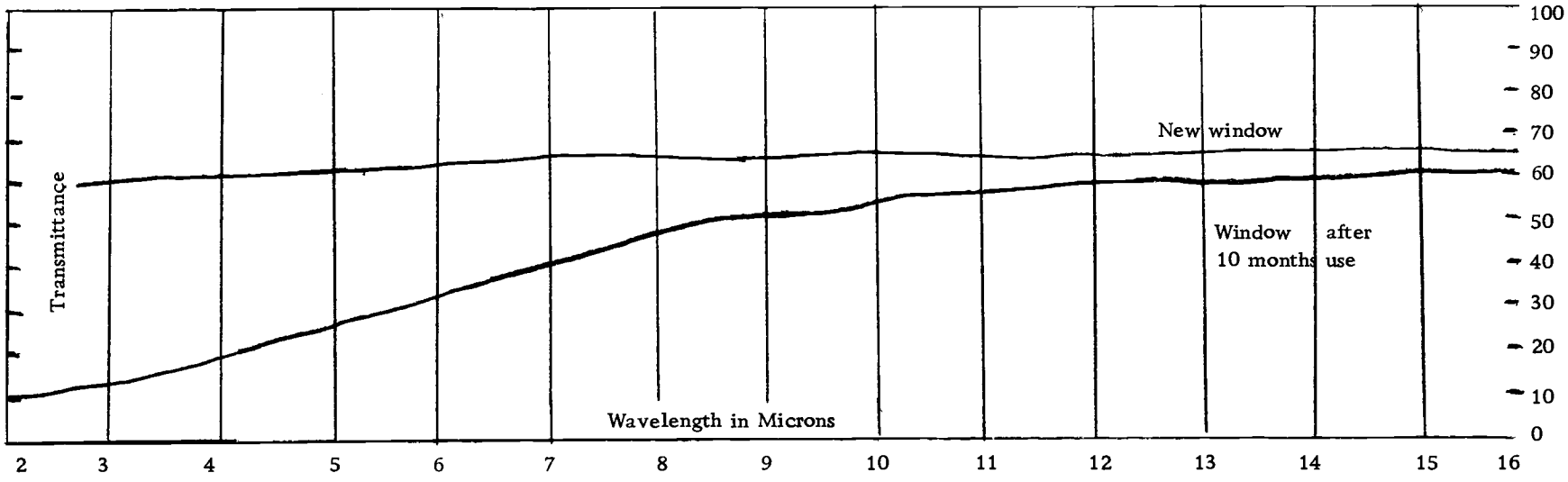
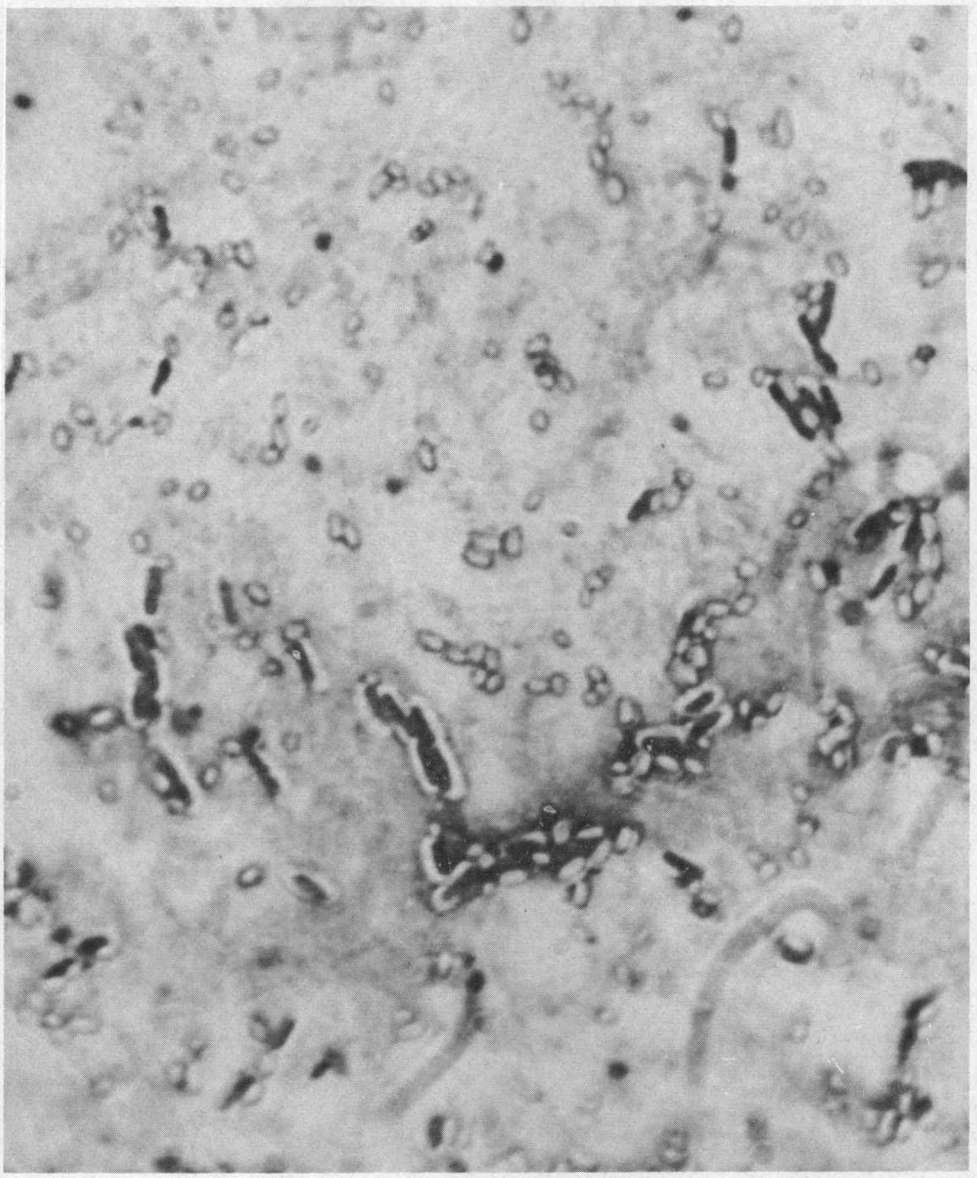


Figure 2. The effect of age on the absorption of the silver chloride windows

Figure 3. Cl. butyricum before purification (69% spores)

Heat fixed smear, stained for one minute with crystal violet.



RESULTS

Reproducibility of IR Spectra

Before any work could be done with the spectra of different strains, or of different organisms, it was necessary to determine the degree of reproducibility which could be attained. This was done by selecting certain organisms and repeatedly making infra-red absorption spectra of thin smears of different cultures of these organisms. These spectra were then compared with each other and any variations or regions of variation were noted. The results are shown in Figures 4, 5, 6, and 7.

It is seen, upon examination of these spectra, that certain regions are subject to more variation than others. A great deal of variation is seen in the region from 6.0 μ to 6.8 μ. This region is associated with the carbon oxygen and carbon nitrogen bonds of the type found to a large extent in proteins. Two very large absorption peaks are seen in this area, and the variation that is seen is due to a distortion of these peaks, which have a greater amount of absorption than the machine is able to accurately measure on the thicker smears. A high degree of uniformity is seen in this area when spectra are made from very thin smears. (See Figures 19-24.) A small amount of variation is also seen in the region from 6.8 μ to 7.0 μ,

Figure 4. The reproducibility of absorption spectra

<u>Clostridium botulinum</u> non-toxigenic "E-like" Strain 15Ati. Two day old cultures on TPGY agar. Films dried one day in an evacuated desiccator over P_2O_5. Note the variation from 8.5 μ to 10.0 μ, and from 6.0 μ to 6.8 μ. The variation from 6.0 μ to 6.8 μ is due to differences in smear thickness. #60 and #61 were made from the same culture on the same day, and #170 and #171 were made from a different culture on a different day. 54 was made one day before 60 and 61.

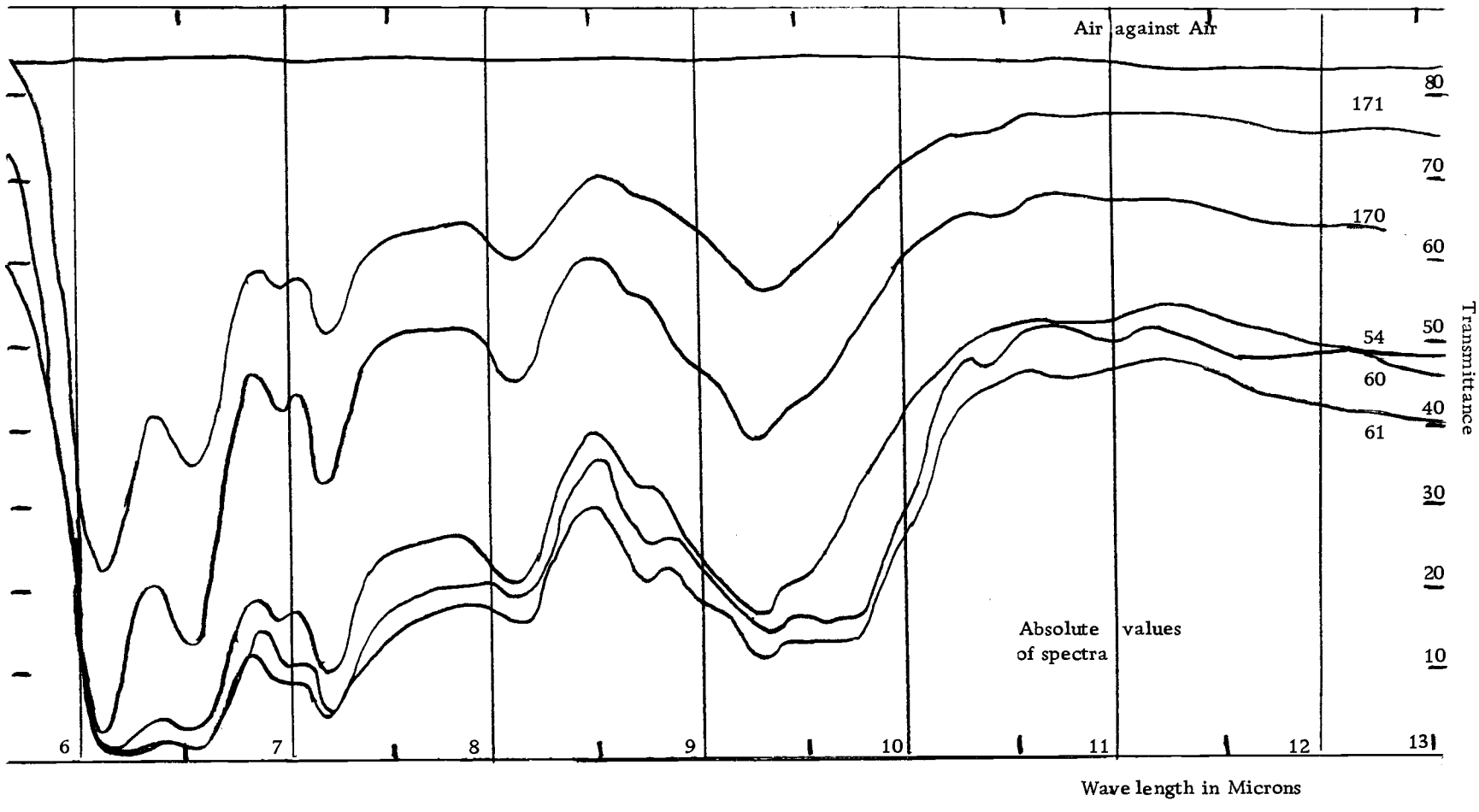

Air against Air
171
170
54
60
61
80
70
60
50
40
30
20
10
Transmittance
Absolute values
of spectra
6
7
8
9
10
11
12
13
Wave length in Microns

Figure 5. The reproducibility of absorption spectra

Clostridium botulinum nontoxigenic "E-like." Strain 34-1. Two day old culture on TPGY agar, dried one day in an evacuated desiccator over P_2O_5. Note variation from 10 μ to 12 μ. (The variation from 6.0 μ to 6.8 μ is due to smear thickness.)

#166 and #167 were made from the same culture on the same day, while #51 and #53 were each made from different cultures on different days.

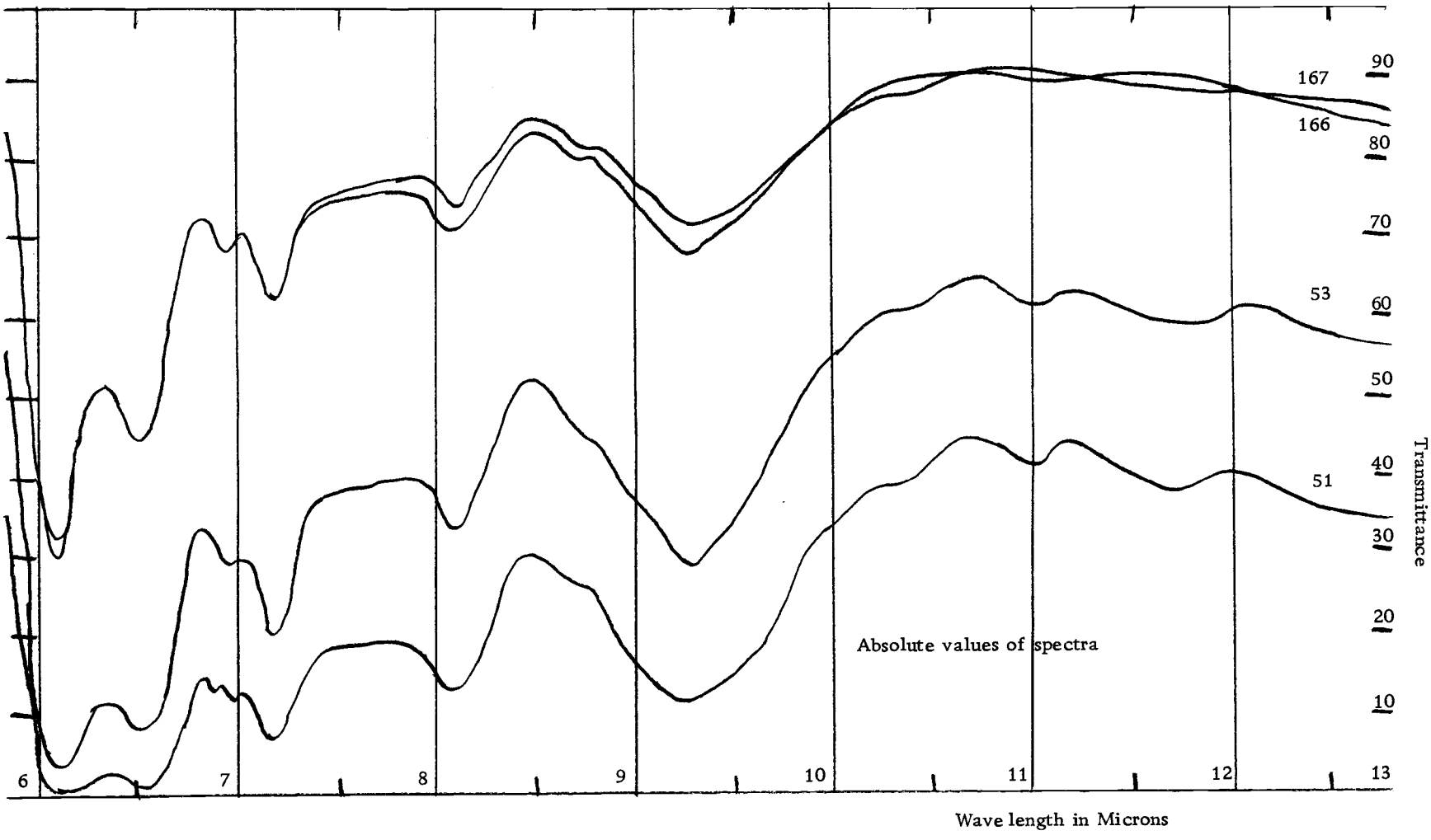

167
166
53
51
90
80
70
60
50
40
30
20
10
13
Transmittance
Absolute values of spectra
6
7
8
9
10
11
12
Wave length in Microns

Figure 6. The reproducibility of absorption spectra

Clostridium botulinum, non-toxigenic "E-like" Strain 066BNT two day old culture on TPGY agar. Dried one day in an evacuated desiccator over P_2O_5. #168 and #169 were made from the same culture on the same day, while #49 and #50 were made from a different culture on a different day.

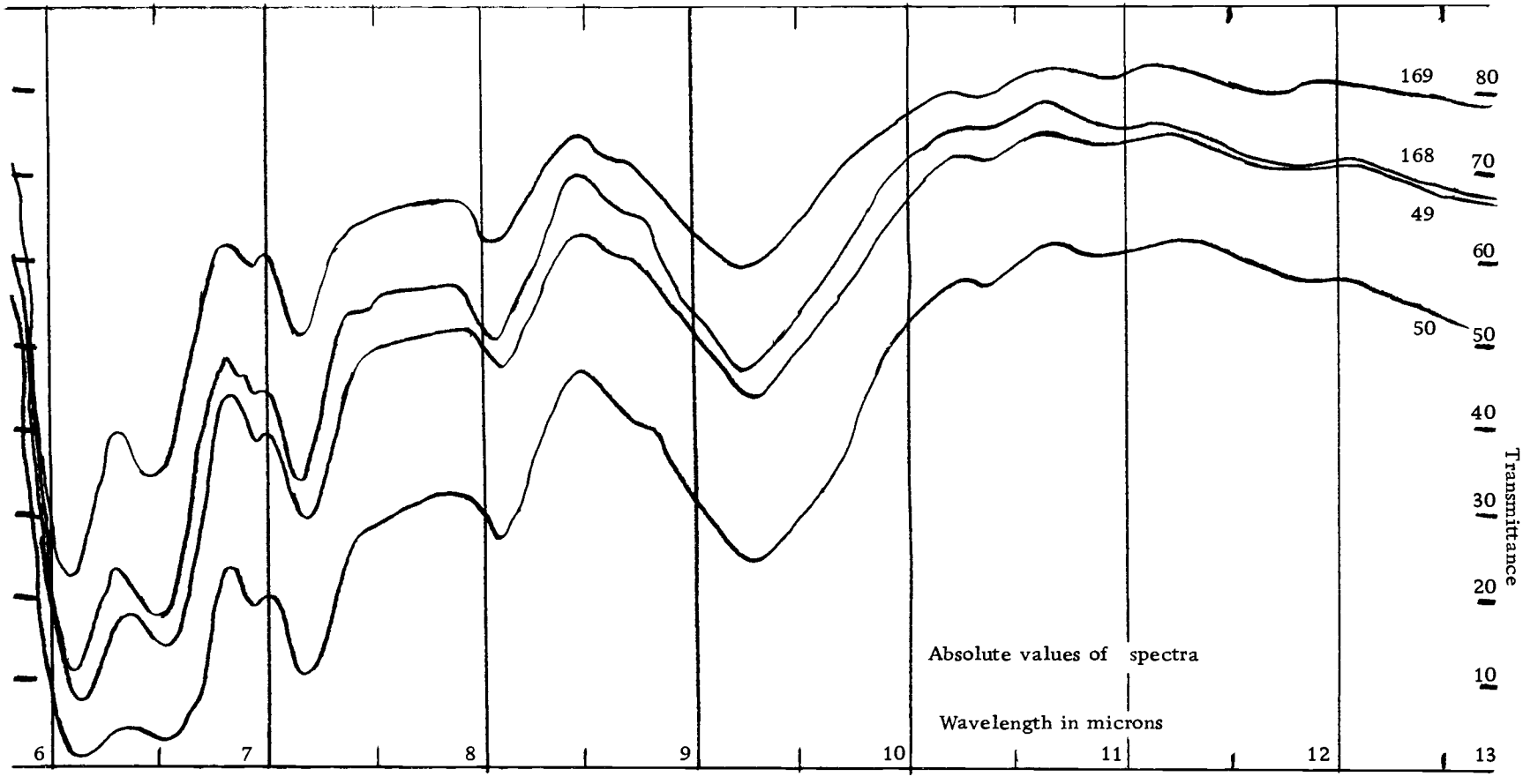
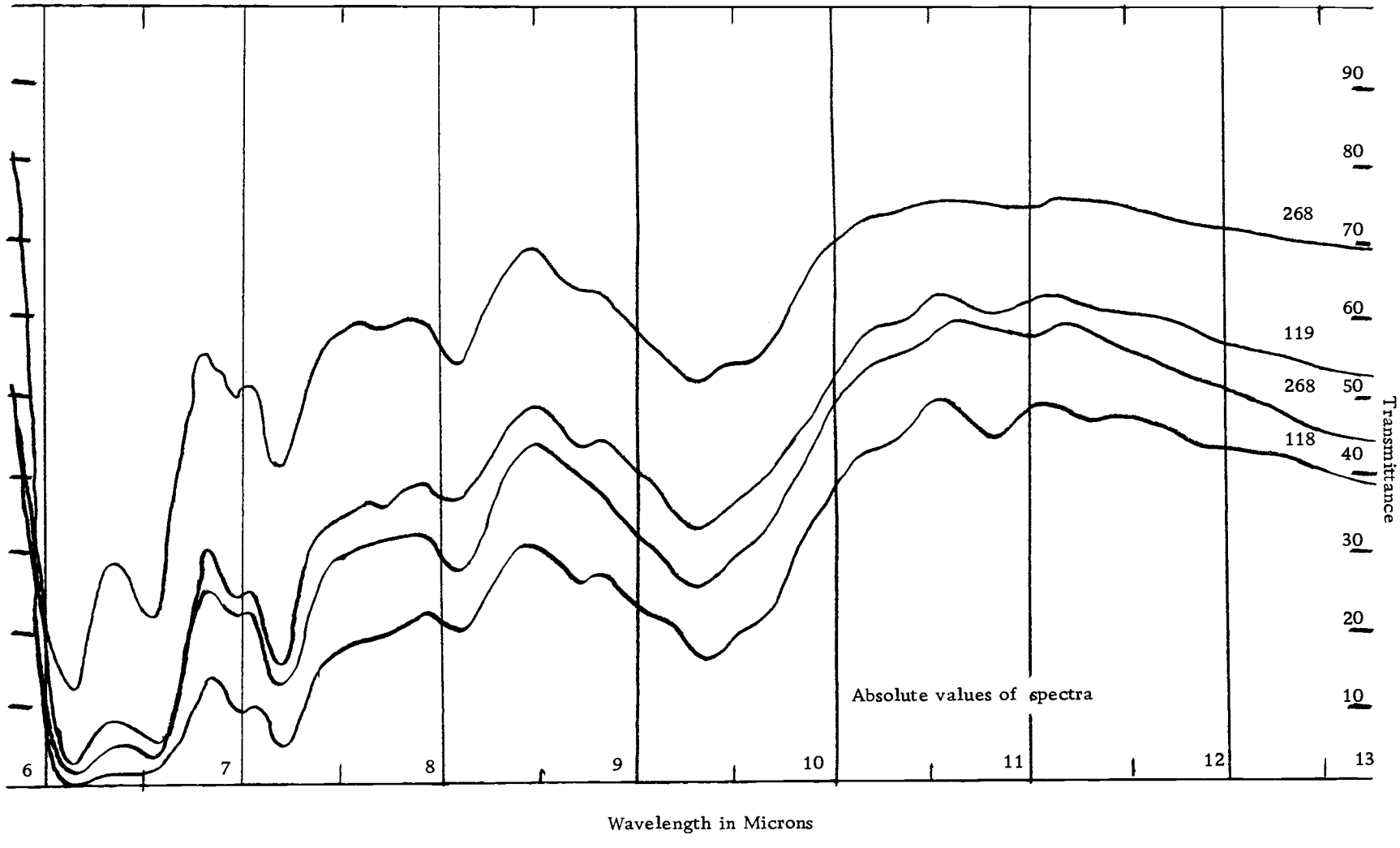

169
168
49
50
80
70
60
50
40
30
20
10
13
Transmittance
Absolute values of spectra
Wavelength in microns
6
7
8
9
10
11
12

Figure 7. The reproducibility of absorption spectra

An illustration of the variation in the spectra of Clostridium botulinum Type F, two day old culture. Note variation from 6.0 μ to 6.8 μ; this is associated with the thickness of the smear (the above are absolute values). Note variation from 10-12 μ. This is due partially to the thickness of the smear and is partially unexplainable. This area is unreliable as an index of spectral type. The two #268s were made on the same day from the same culture, while #118 and #119 were made on a different day from a different culture.


90
80
268
70
60
119
268
50
118
40
30
20
10
Transmittance
Absolute values of spectra
6
7
8
9
10
11
12
13
Wavelength in Microns

making this region unreliable also. The reason for this variation is not immediately apparent. The region from 7.0 μ to 10.0 μ has the best reproducibility, and most of the changes in this region can be attributed to changes produced by the organisms themselves. The most significant results found in this study are seen in this region. Variation was again seen in the region above 10.0 μ, making it unreliable for use in this study.

The Effect of Smear Thickness of the Bacterial Films

Certain things are seen to stand out upon examination of spectra made from bacterial films of different thicknesses. In the thin smear, the peaks tend to be flattened and the whole spectrum seems to be washed out. The exception to this is in the region from 6.0 μ to 6.8 μ. This is a region dominated by two large peaks. The depth of these peaks is such that they are seen to stand out best in a moderately thin smear. Some of the finer (see Figure 8) characteristics in other areas of the spectrum may be distinguished by the acute observer; however, great care must be used, and if at all possible a thicker smear should be prepared. In a smear which is too thick, the region from 6.0 μ to 7.5 μ becomes totally indistinguishable, the region from 7.5 μ to 10.0 μ becomes flattened, but the region above 10.0 μ seems to give good representation. The region above

10.0 μ is unreliable from a point of reproducibility (see Figures 4-7), therefore, the best smears tend to absorb in the range from 20% to 70% transmittance, as is shown in Figure 8. Small inflections in the region from 7.0 μ to 10.0 μ are best seen in this range, and this is the range from which most of the information in this study has been obtained.

Spectral Patterns and Distribution

It was found that the most reproducible section of the IR spectrum, which would also give unique and somewhat characteristic variation was in the region from 7.0 μ to 10.0 μ. It was found, in addition, that these characteristic variations were magnified to the greatest extent when the 7.0 μ to 10.0 μ region had its entire transmittance lying in the range from 20% to 70%.

When smears of the clostridia within the region and range described above were examined, four unique and characteristic patterns were discovered. In addition to this, two unique and rare patterns could be seen. When these six patterns were compared with the IR spectral patterns of Sarcina lutea, Staphylococcus aureus, Bacillus subtilis, and Bacillus megatherium, they were seen to be unique and distinguishable from the spectra of these four organisms. These patterns seem to vary, not only with the strain, but also with the physiological state of the strain.

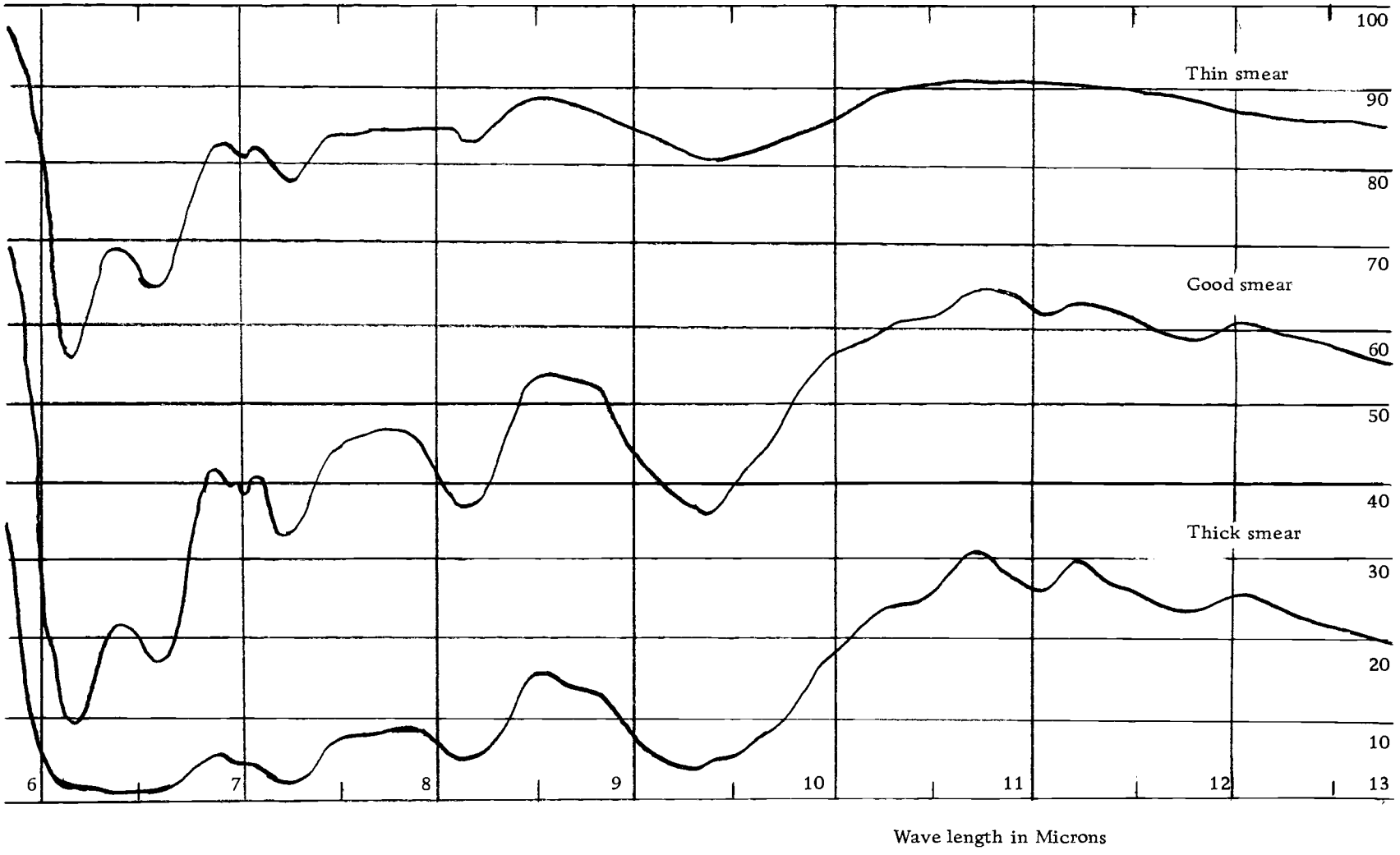


Figure 8. The effect of smear thickness on the spectra

Pattern α (Figure 9) is characterized by a sharp peak at 9.2 μ to 9.3 μ, and the almost complete absence of a peak at 8.7 μ. The trough at 8.4 μ to 8.5 μ tends to be rather pointed, as in spectrum #65, but may vary from the beginnings of the 8.7 μ peak as in #81, to a flattened 8.4 μ to 8.7 μ trough as in figures #146 and #152. It will be recalled that Wright and Lockhart (1965) state that this type of flattening can be caused by changes in the state of the cells, such as the division rate or the limiting substrate. Since this variation could not be correlated with the other spectral patterns very well, it was not chosen as a pattern characteristic.

Pattern β (Figure 10) is quite unique, and is distinguished by a sharp pointed trough at 8.4 μ to 8.5 μ, a peak at 8.7 μ, a peak at 9.2 μ to 9.3 μ, and a plateau or flat area from 9.4 μ to 9.75 μ. In extreme cases, a third peak may form 9.75 μ. This type of pattern is caused by the storage of a large amount of glycogen in the cell (Levine, 1953b, c).

Pattern γ (Figure 11) is characterized by a trough at 8.4 μ to 8.5 μ, a peak at 8.7 μ, and a peak at 9.3 μ.

Pattern δ (Figure 12) is characterized by a slight depression at 8.5 μ to 8.6 μ, and at 9.0 μ to 9.1 μ. This tends to give the sloping line from 8.5 μ to 9.3 μ an undulating appearance. Pattern δ is very similar to pattern α, and in some cases, they almost seem to merge.

Patterns ε and λ (Figure 13) are quite unique. They are characterized by a very reduced peak at 8.1 μ, the trough at 8.5 μ,

an abrupt change in slope at 9.0 μ to 9.1 μ, a peak at 9.3 μ, and a small plateau from 9.4 μ to 9.5 μ. In addition, the region from 10.0 μ to 11.5 μ seems to have a pronounced wavy pattern. Pattern λ may be distinguished from pattern ε by the appearance of a small peak at 8.7 μ.

Pattern G (Figure 14) has a very rounded peak from 9.0 μ to 10.0 μ. This pattern is from Bacillus subtilis. Notice that it may be distinguished from all other patterns previously given.

Pattern H is very different yet (see Figure 15). It is distinguished by a sharp peak at 5.8 μ, a peak at 7.7 μ, a peak at 8.5 μ, a peak at 8.9 μ, a wavy plateau from 9.2 μ to 9.4 μ, and a peak at 9.5 μ. This pattern results from the accumulation of large amounts of β hydroxy butyric acid in the cells (Blackwood and Agnes, 1957; Haynes et al., 1958). Many members of the genus Bacillus give this type of pattern.

Sarcina lutea gives the type of pattern as shown in Figure 16 (Pattern I). It is similar to pattern G, (Figure 14), but the area from 9.0 μ to 10.0 μ is not as smooth. There also seems to be a trough at 10.6 μ.

Pattern J is given by Staphylococcus aureus. It has a depression from 7.4 μ to 7.8 μ, a strong peak at 8.1 μ, and a flattened area from 8.5 μ to 8.8 μ.

The distribution of organisms into these various patterns at

Figure 9. Spectral pattern α

Spectral #	Organism	Age
	Cl. botulinum Type E and "E-like" strains	
#81	Tuna Can (toxic)	2 day
#168	066BNT (nontoxic)	2 day
#65	170IX (nontoxic)	2 day
#163	805Bb (nontoxic)	2 day
#146	Saratoga (toxic)	2 day
#152	070 (toxic)	2 day

All organisms were grown on TPGY agar. Note the deeper peak at 8.1 μ in type α (P=0 absorbs here). Also note the absorption peak at 9.25 μ, and the beginnings of an absorption peak at 8.7 μ in #81.

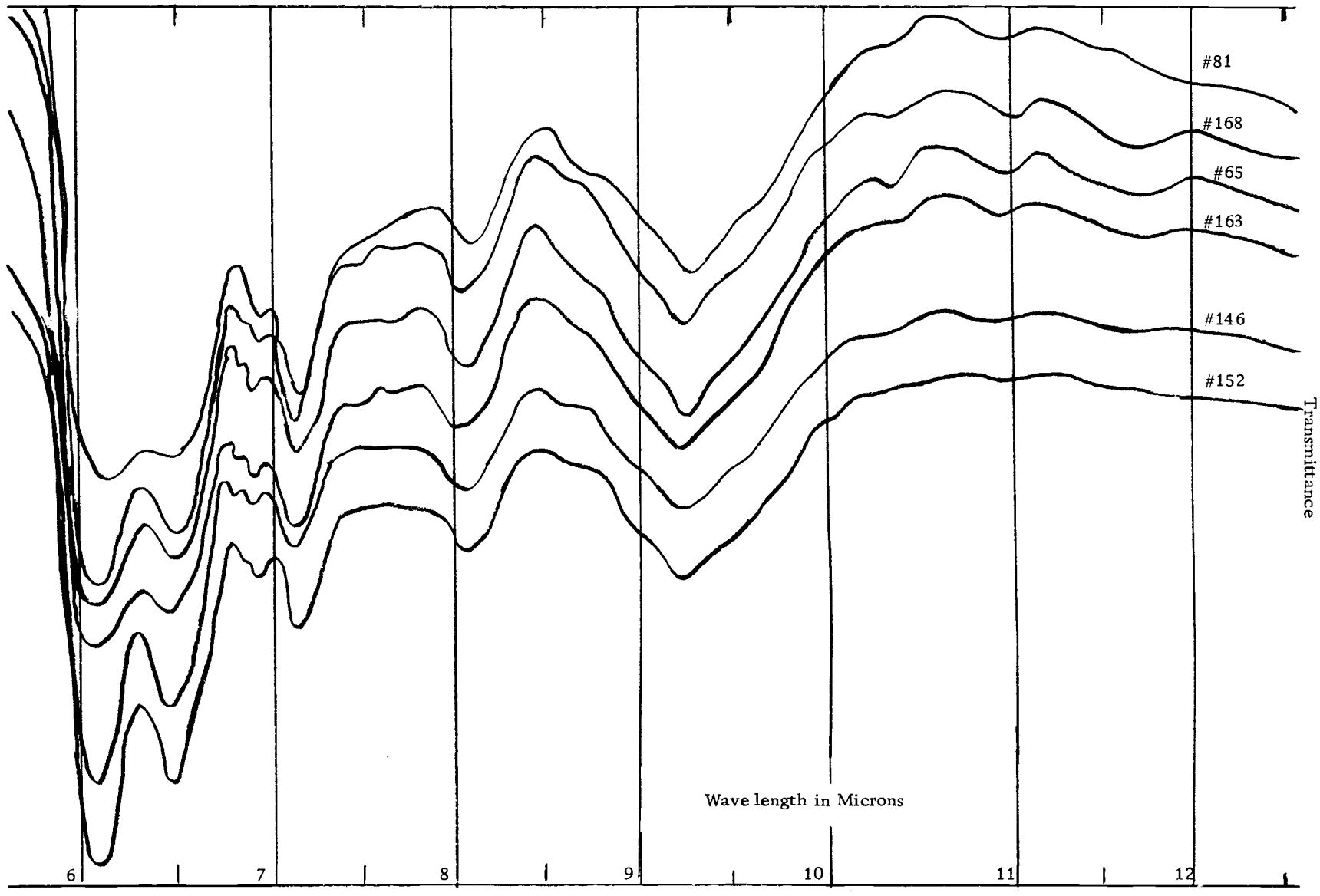

#81
#168
#65
#163
#146
#152
Transmittance
Wave length in Microns
6
7
8
9
10
11
12

Figure 10. Spectral pattern β

Spectral #	Organism	Age
	Cl. botulinum Type E and "E-like" strains	
#222	GB-3 (nontoxic)	6 day
#60	15Ati (nontoxic)	2 day
#150	VH#1 (toxic)	2 day
#156	VH#2 (toxic)	2 day
#142	Beluga (toxic)	2 day
#273	Cl. butyricum	2 day

All cultures were grown on TPGY agar. Spectral pattern β is characterized by the presence of stored glycogen (Levine et al., 1953). Note the absorption peaks at 8.7 μ, 9.25 μ, and 9.75 μ.

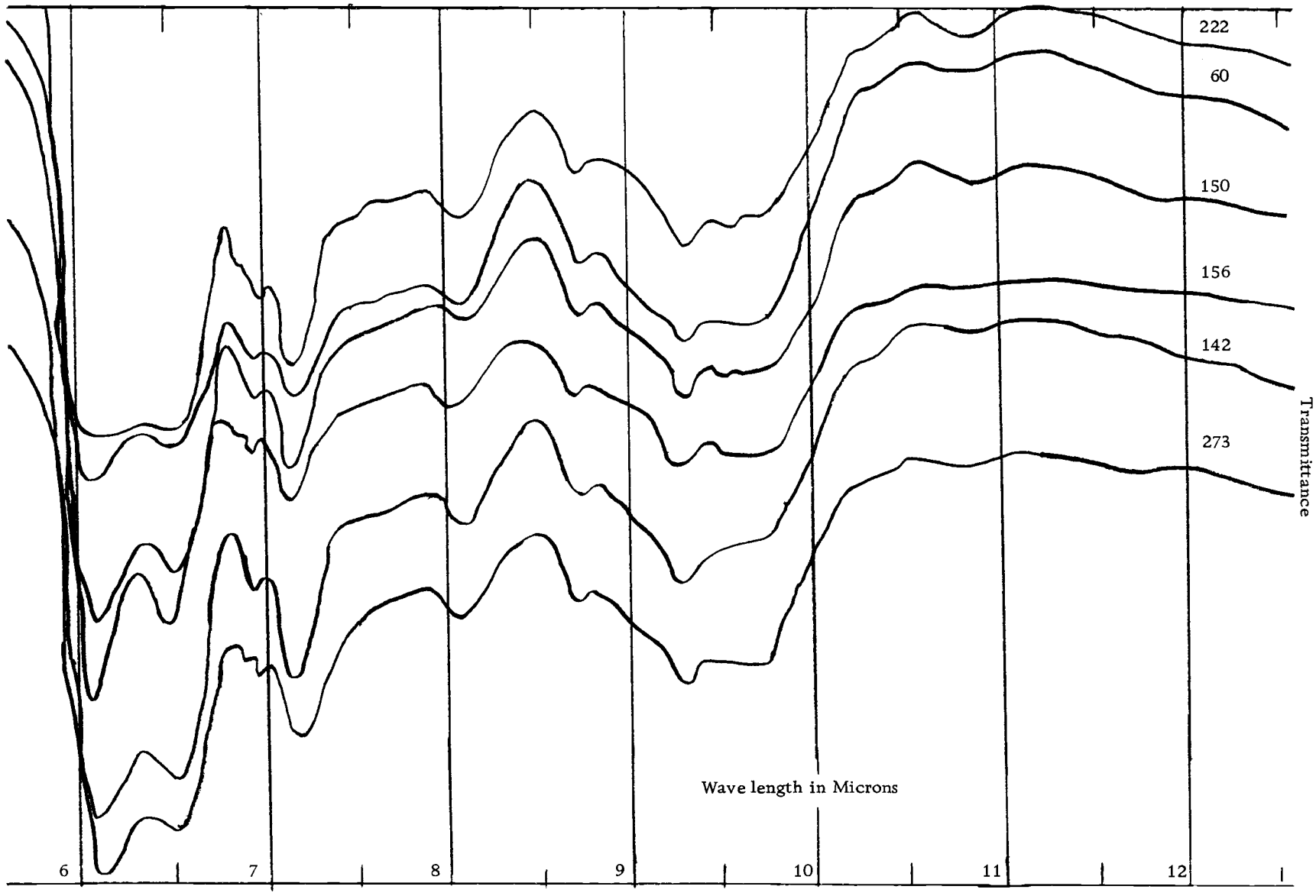

222
60
150
156
142
273
Transmittance
Wave length in Microns
6
7
8
9
10
11
12

Figure 11. Spectral pattern ?

Spectral #	Organism	Age
	Cl. botulinum "E-like" strains	
#179	GB-3 (nontoxic)	2 day
#165	170IX (nontoxic)	2 day
#73	8Ati (nontoxic)	2 day
#76	15CBT (nontoxic)	2 day
#215	170IX (nontoxic)	2 day
#224	805Bb (nontoxic)	2 day

All cultures were grown on TPGY agar. Note the development of a peak at 8.7 μ.

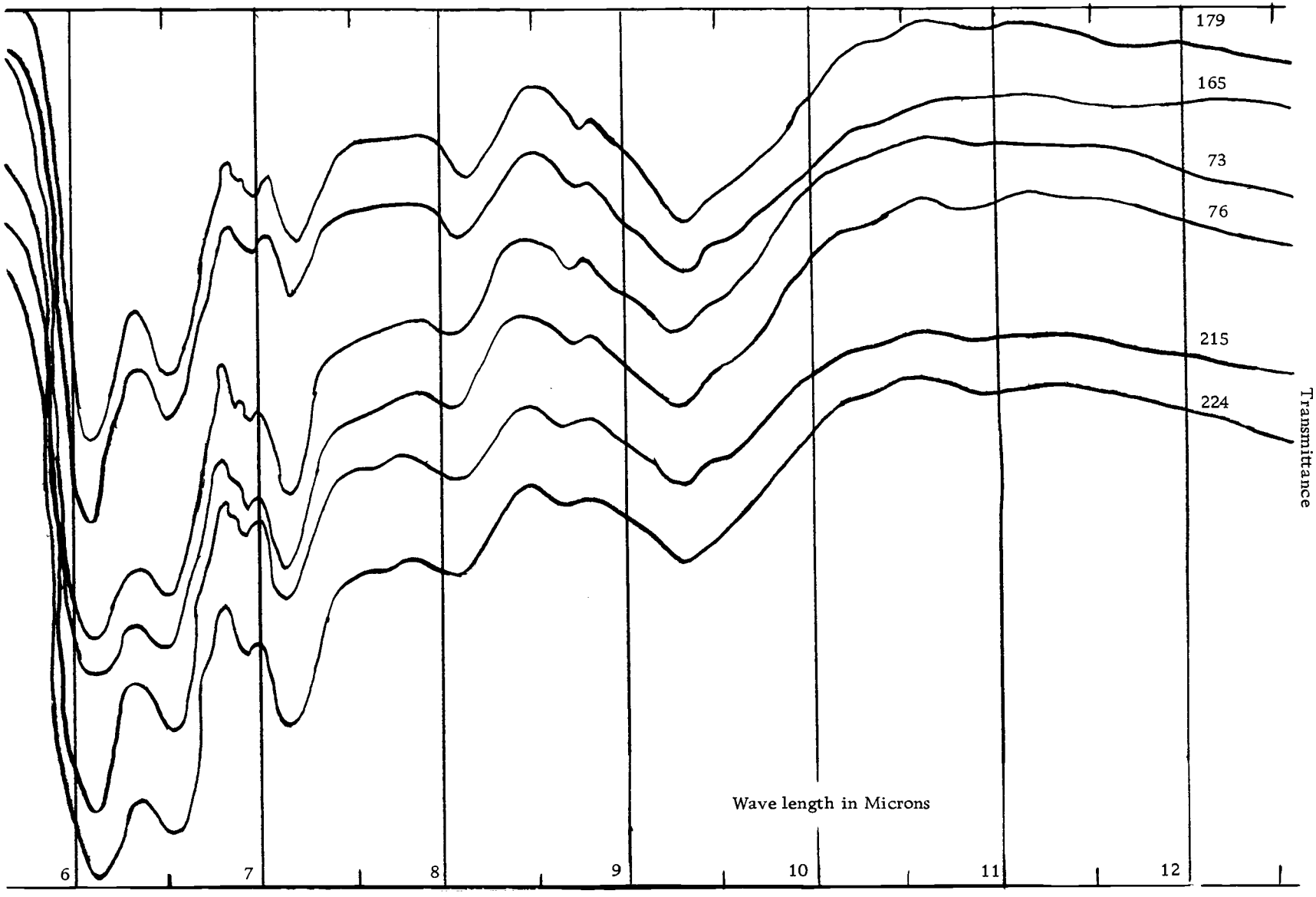

179
165
73
76
215
224
Transmittance
Wave length in Microns
6
7
8
9
10
11
12

Figure 12. Spectral pattern δ

Spectral #	Organism	Age
	Cl. botulinum Type B strains	
#129	115B	5 day
#254	113B	5 day
	Type A strains	
#137	33A	5 day
#249	5A	5 day
#302	Cl. sporogenes	5 day
#260	Cl. welchii	5 day

All cultures grown on TPGY agar.

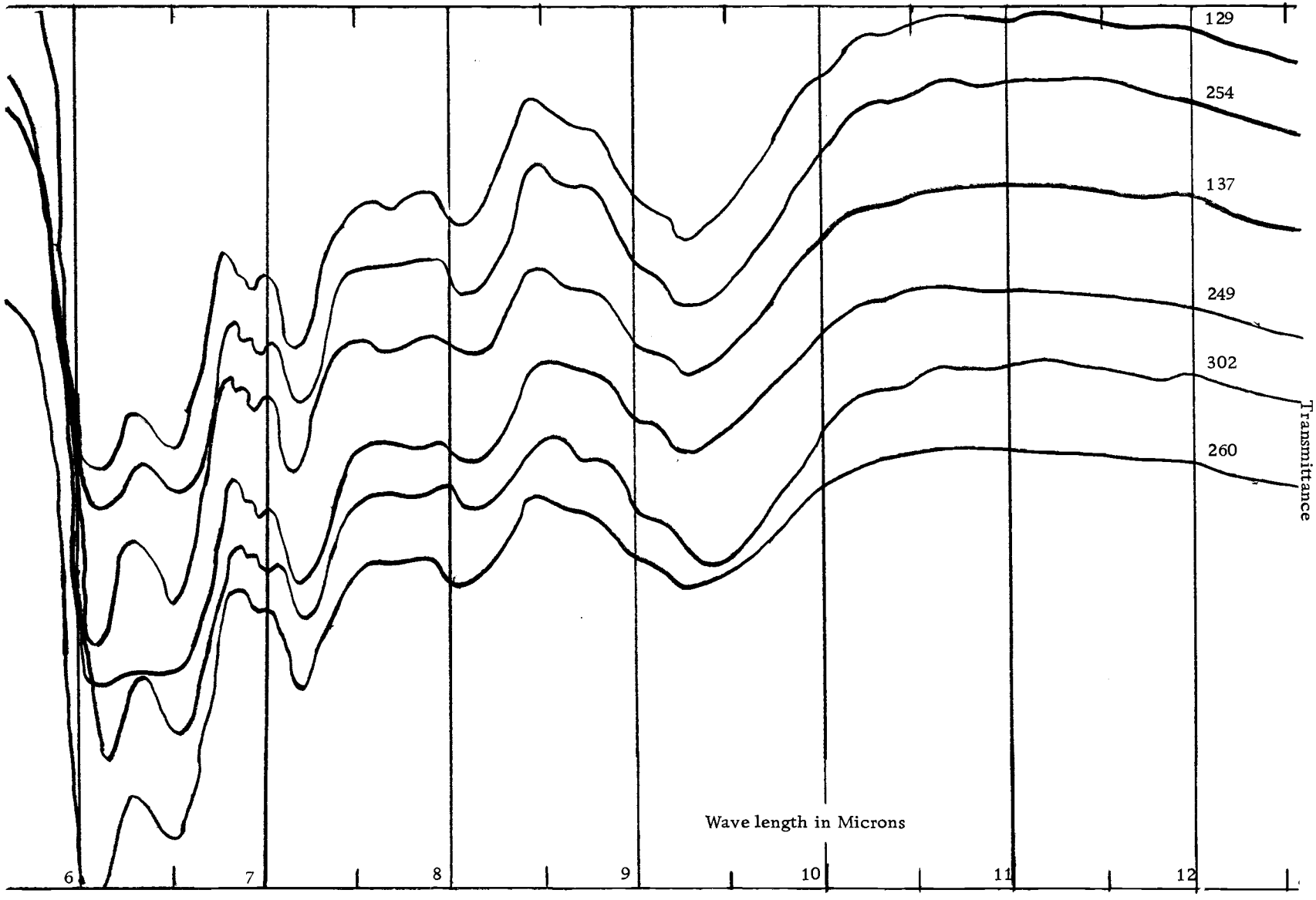

129
254
137
249
302
260
Transmittance
Wave length in Microns
6
7
8
9
10
11
12

Figure 13. Spectral patterns ε and λ

Spectral #	Organism	Age
#245	Cl. bifermentans	2 day
#78	Cl. sporogenes	2 day
#77	Cl. sporogenes	2 day
	Cl. botulinum	
	Type E strain	
#180	066 BNT (nontoxic)	5 day
#181	066 BNT (nontoxic)	5 day

All cultures were grown on TPGY agar. #245 is pattern ε, and all of the others are pattern λ.

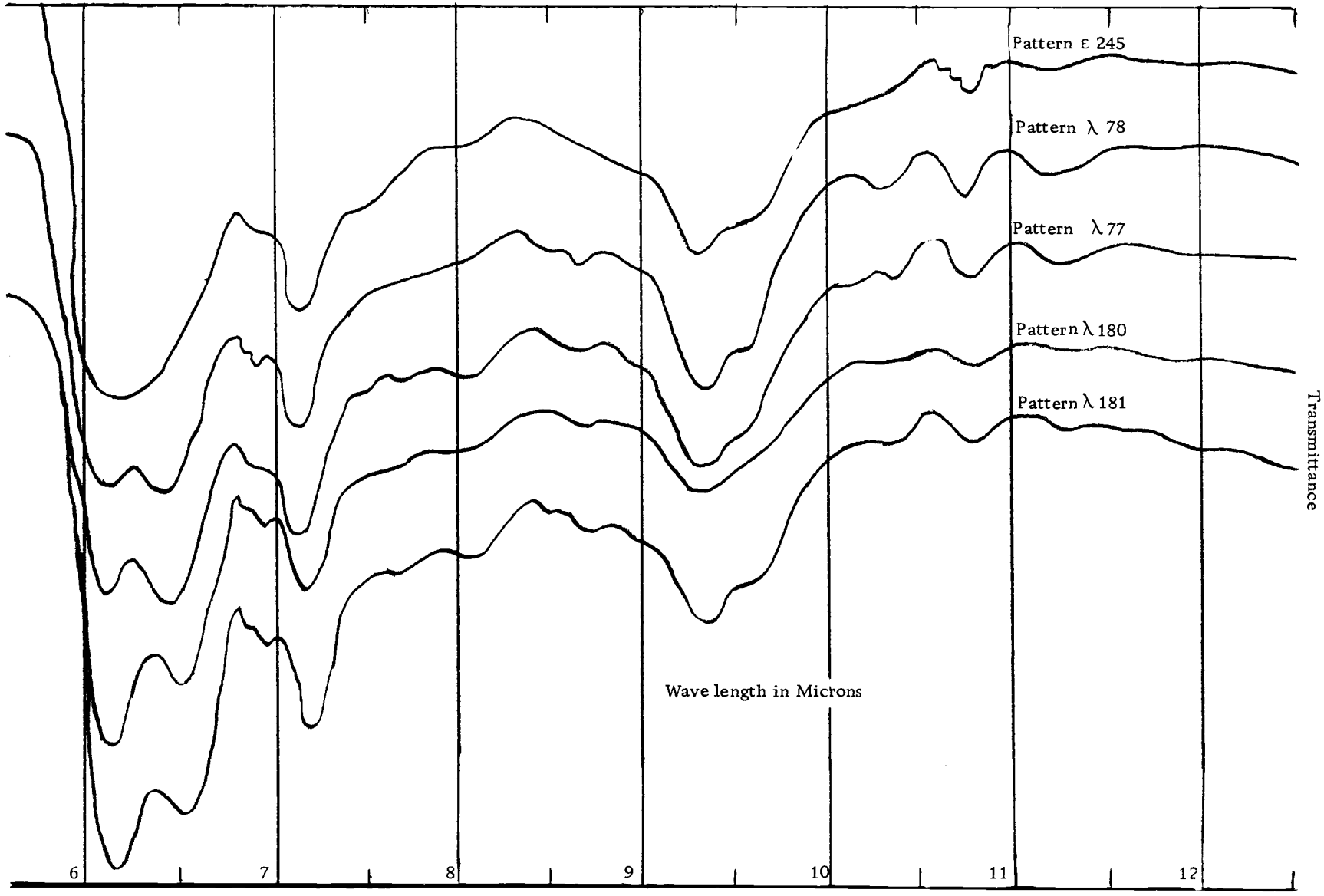

Pattern ε 245
Pattern λ 78
Pattern λ 77
Pattern λ 180
Pattern λ 181
Transmittance
Wave length in Microns
6
7
8
9
10
11
12

Figure 14. Spectral pattern G

Spectral #	Organism	Age
#338	Bacillus subtilis	2 day
#338	Bacillus subtilis	2 day
#345	Bacillus subtilis	2 day
#345	Bacillus subtilis	2 day

All cultures were grown on TPGY agar. The two #338s were smeared from the same culture while the two #345s were smeared from a different culture.

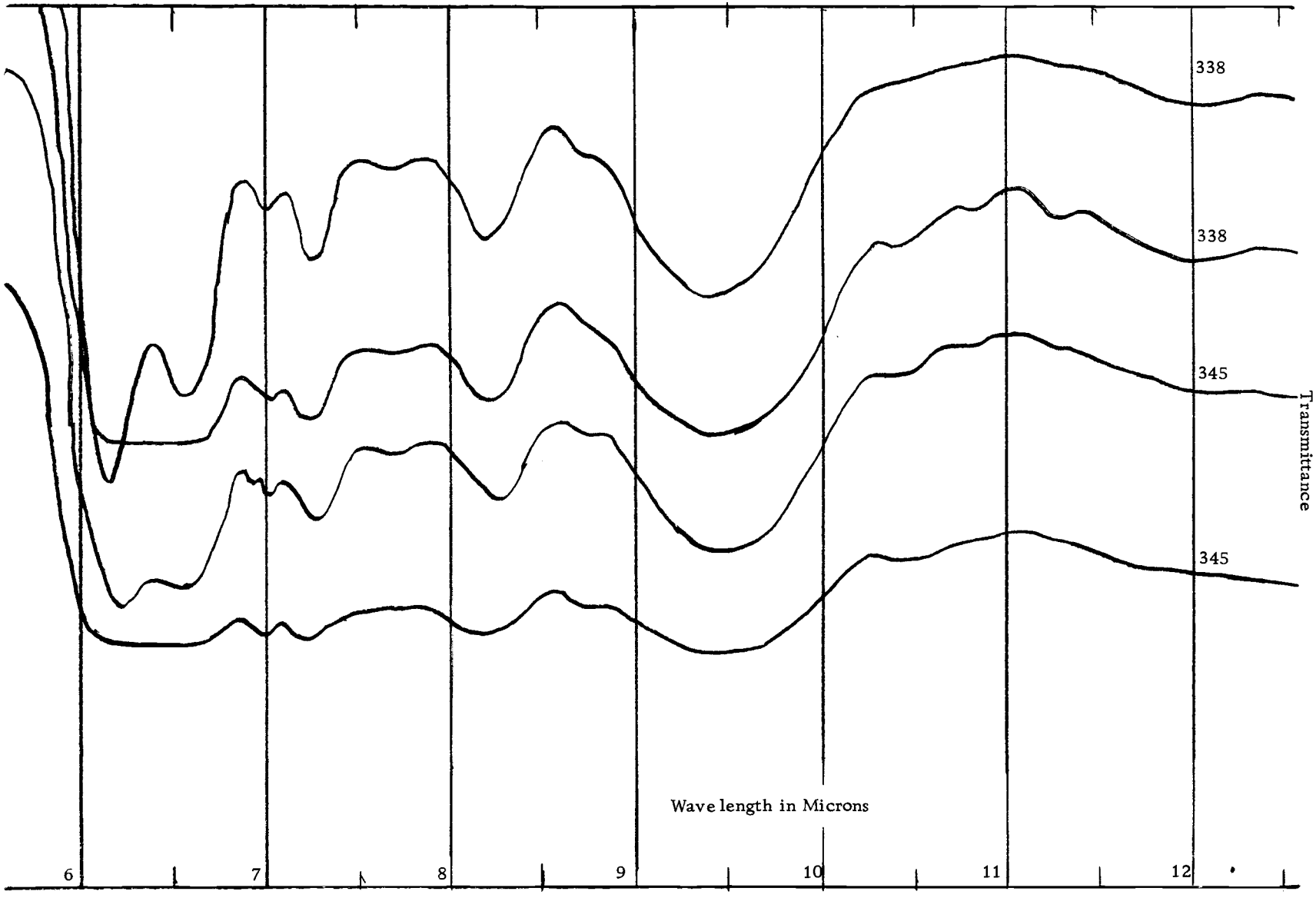

338
338
345
345
Transmittance
Wave length in Microns
6
7
8
9
10
11
12

Figure 15. Spectral pattern H

Spectral #	Organism	Age
#344	Bacillus megatherium	2 day

The culture was grown on TPGY agar. The spectral pattern is due to the formation of β Hydroxy Butyric Acid in the cells.

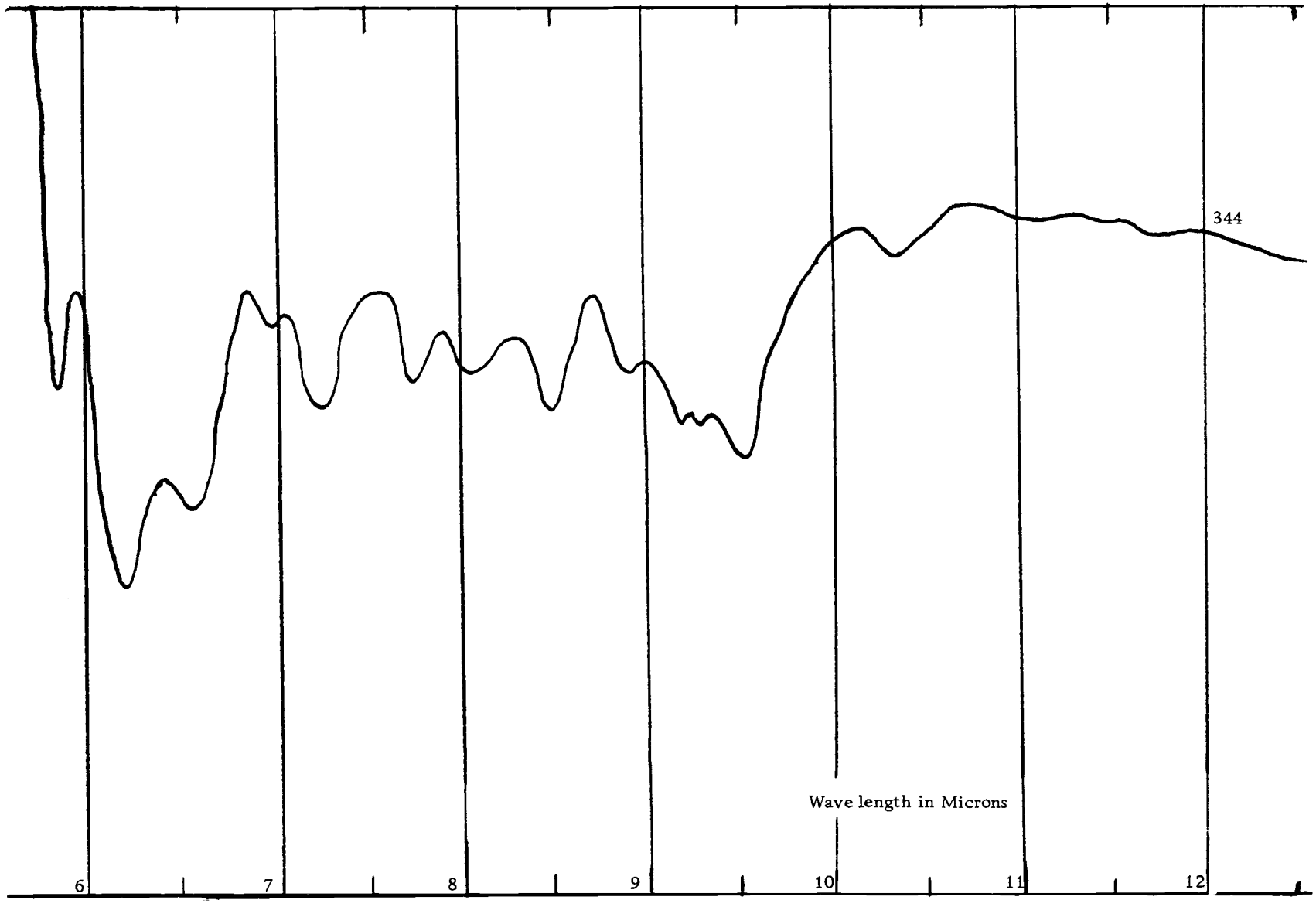

344
Transmittance
Wave length in Microns
6
7
8
9
10
11
12

Figure 16. Spectral pattern I

Spectral #	Organism	Age
#346	Sarcina lutea	2 day
#346	Sarcina lutea	2 day
#341	Sarcina lutea	2 day
#341	Sarcina lutea	2 day

All cultures were grown on TPGY agar. The first two smears were made from the same culture, while the last two smears were made from a different culture.

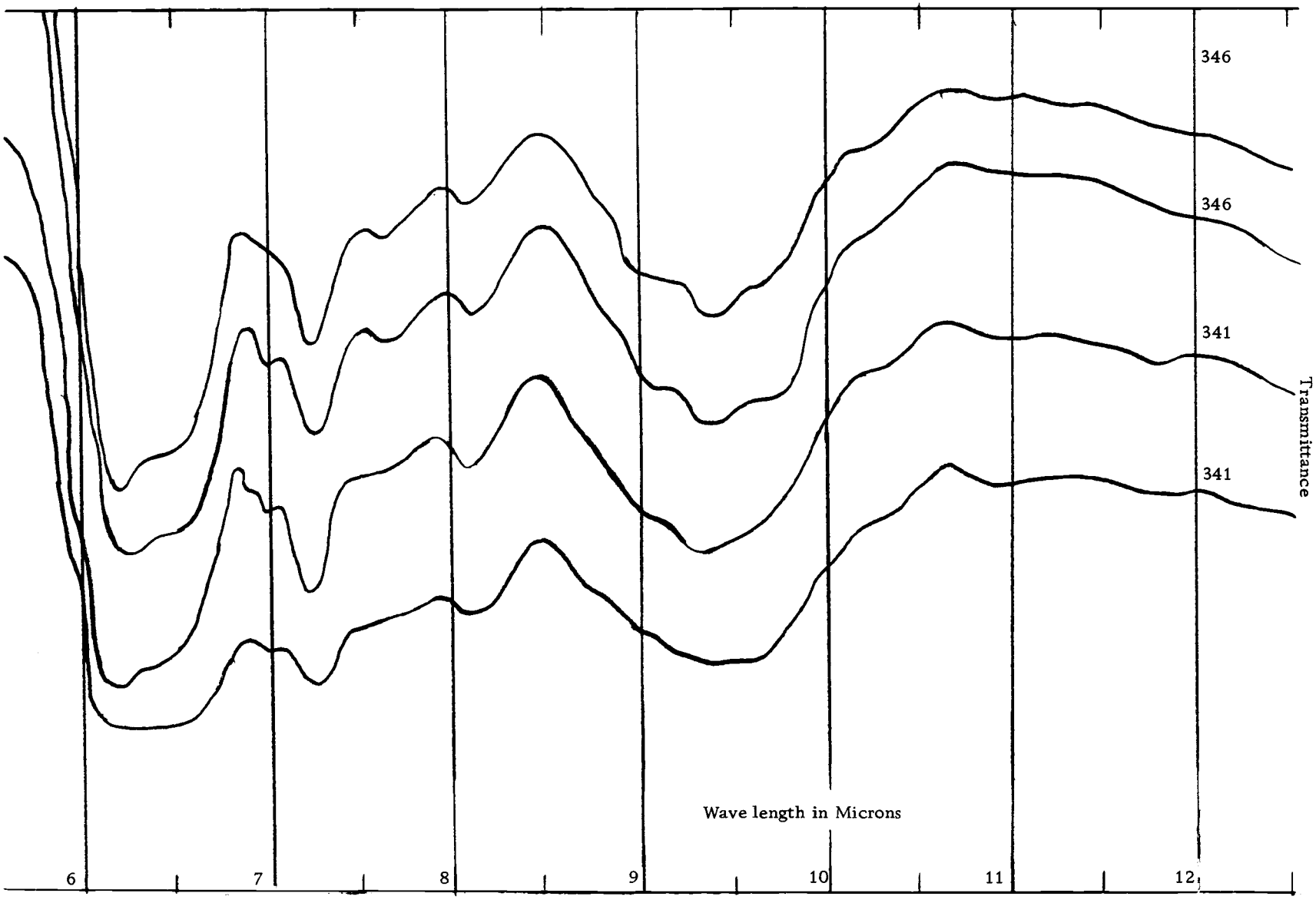

346
346
341
341
Transmittance
Wave length in Microns
6
7
8
9
10
11
12

Figure 17. Spectral pattern J

Spectral #	Organism	Age
#337	Staphylococcus aureus	2 day
#337	Staphylococcus aureus	2 day
#342	Staphylococcus aureus	2 day
#342	Staphylococcus aureus	2 day

All cultures were grown on TPGY agar. The first two smears were made from the same culture, while the last two smears were made from a different culture.

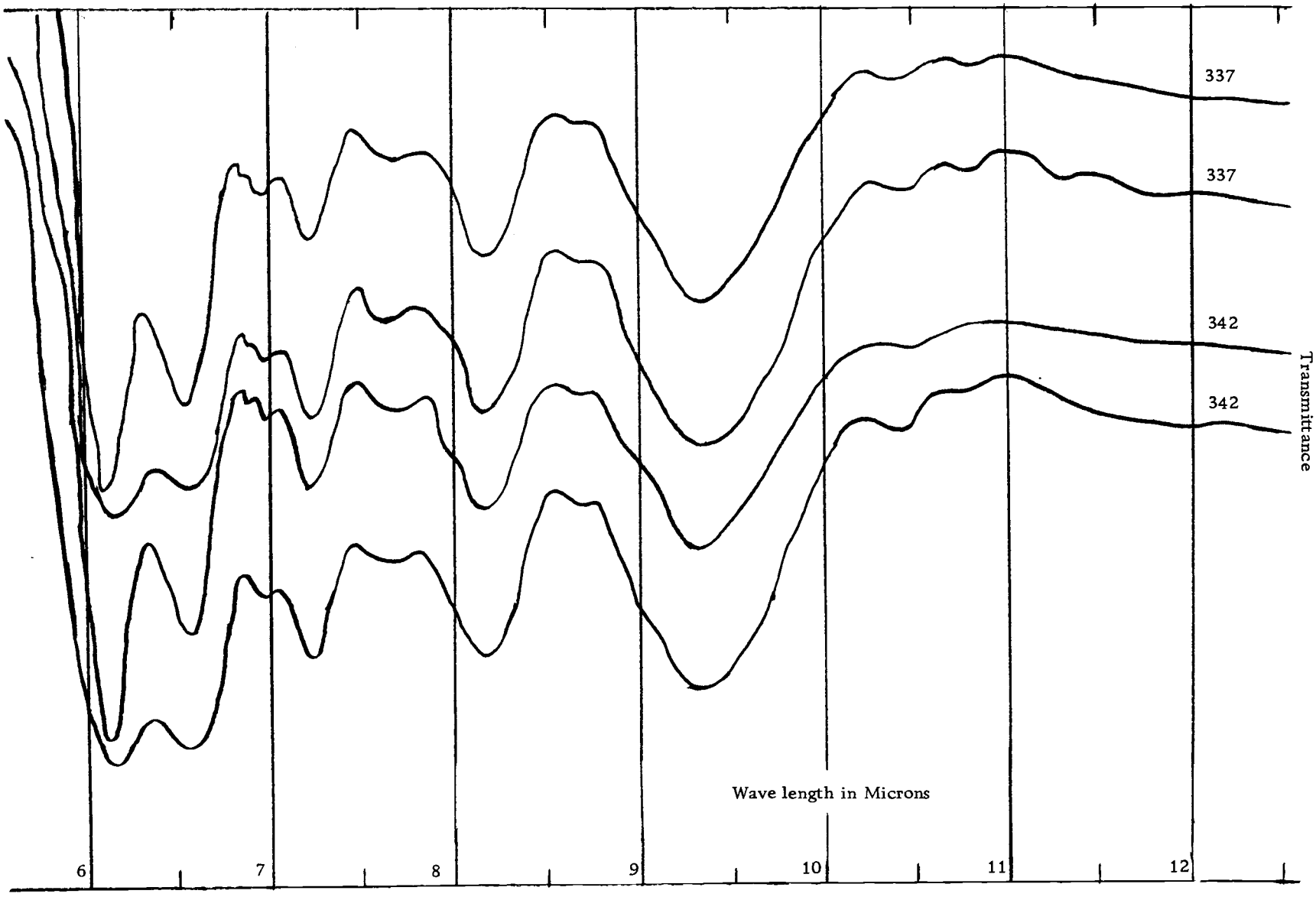

337
337
342
342
Transmittance
Wave length in Microns
6
7
8
9
10
11
12

Table 1. Spectral patterns of the two day old toxic strains of Cl. botulinum type E

Strain	Spectrum #	Spectral pattern
VH#1	151	β
VH#1	150	β
VH#1	79	β
Beluga	143	β
Beluga	142	β
Beluga	86	β
VH#2	157	β
VH#2	156	β
VH#2	90	β
Tuna Can	145	α
Tuna Can	81	α
Tuna Can	147	α
070	83	α
070	152	α
070	153	α
Isolate #3 (OSU)	87	α
Isolate #3 "	158	α
Isolate #3	159	α
Isolate #1 (OSU)	92	α
Isolate #1 "	160	α
Isolate #1 "	161	α
Saratoga	82	α
Saratoga	146	α
Saratoga	144	α
066B	80	α
066B	149	α
066B	148	α
Detroit	242	α
Detroit	139	α
Detroit	85	α
Detroit	241	α
Isolate #2 (OSU)	239	α
Isolate #2 "	138	α
Isolate #2 "	240	α
Isolate #2 "	88	α
Alaska	91	α
Kalamazoo	84	α
Kalamazoo	155	α
Kalamazoo	154	α
Iwanai	89	β
Iwanai	140	α
Iwanai	141	α

Table 2. Spectral patterns of the two day old toxic strains of Clostridium botulinum, types A, B, and F

Strain	Spectrum #	Spectral pattern
Type F	268	γ (2 spectra)
Type F	118	γ
Type F	119	ν
Type B		
115B	114	δ
115B	267	δ
113B	266	δ
113B	120	δ
113B	121	δ
113B	115	δ
Type A		
5A	117	δ
5A	264	δ
5A	263	δ
33A	265	δ
33A	116	δ

Table 3. Spectral patterns of the two day old nontoxic strains closely resembling Clostridium botulinum, type E

Strain	Spectrum #	Spectral pattern
170C	173	α
170C	172	α
066BNT	169	α
066BNT	168	α
34-1	167	α
34-1	166	α
S-9	174	α
S-9	70	α
S-9	69	α
15CBT	75	γ
15CBT	76	γ
8Ati	74	γ
8Ati	73	γ
GB-3	179	γ
GB-3	178	γ
GB-3	72	γ
GB-3	71	γ
	Variable strains	
900D	176	α
900D	177	α
900D	68	γ
900D	67	γ
15Ati	171	α
15Ati	170	α
15Ati	60	β
15Ati	61	β
805Bb	163	α
805Bb	163	α
805Bb	63	γ
805Bb	64	γ
170IX	65	α
170IX	66	γ
170IX	165	γ

Table 4. Spectral patterns of the two day old strains of the miscellaneous clostridia

Strain	Spectrum #	Spectral pattern
Cl. welchii	288	α (2 spectra)
Cl. welchii	271	α (2 spectra)
Cl. welchii	246	α
Cl. fallax	312	α (2 spectra)
Cl. fallax	277	α (2 spectra)
Cl. novyi	289	α (2 spectra)
Cl. tertium	313	α (2 spectra)
Cl. tertium	275	α (2 spectra)
Cl. tetani	248	α
Cl. tetani	247	α
Cl. tetani	276	α (2 spectra)
	Variable strains	
Cl. sporogenes	274	α (2 spectra)
Cl. sporogenes	269	α (2 spectra)
Cl. sporogenes	113	α
Cl. sporogenes	112	α
Cl. sporogenes	77	λ
Cl. sporogenes	78	λ
Cl. septicum	270	α (2 spectra)
Cl. septicum	244	β
Cl. butyricum	93	α
Cl. butyricum	94	α
Cl. butyricum	273	β (2 spectra)
Cl. bifermentans	111	α
Cl. bifermentans	110	α
Cl. bifermentans	245	ε
Cl. histolyticum	272	α
Cl. difficile	278*	α

*3 day culture

Table 5. Spectral patterns of the two day miscellaneous bacteria

Strain	Spectrum #	Spectral pattern
Neisseria catarrhalis	347	a (2 spectra)
Neisseria catarrhalis	340	a "
E. coli	343	a "
E. coli	336	a "
Sarcina lutea	346	I "
Sarcina lutea	341	I "
Staphylococcus aureus	337	J "
Staphylococcus aureus	342	J "
Bacillus subtilis	338	G "
Bacillus subtilis	345	G "
	Variable	
Bacillus megaterium	339	G "
Bacillus megaterium	344	H "

Table 6. Spectral patterns of the five day old toxic strains of Clostridium botulinum, type E

Strain	Spectrum #	Spectral pattern
Beluga	197	β
Beluga	107	β
Beluga	198	β
VH#1	95*	β
VH#1	187	β
VH#1	188	β
VH#2	104	β
VH#2	209	β
VH#2	210	β
Detroit	211	γ
Detroit	212	γ
Detroit	106	γ
Isolate #3	102	γ
Isolate #3	203	γ
Isolate #3	204	γ
Isolate #2	201	γ
Isolate #2	123	γ
Isolate #2	202	γ
Iwanai	122	γ
Iwanai	109	γ
Iwanai	199	γ
Iwanai	200	γ
Tuna Can	191	γ
Tuna Can	97*	γ
Tuna Can	192	γ
Kalamazoo	96*	γ
Kalamazoo	189	γ
Kalamazoo	190	γ
066B	98*	γ
066B	182	γ
066B	183	γ
Alaska	108	γ
Alaska	207	γ
Alaska	208	γ
Isolate #1	105	γ
Isolate #1	205	γ
Isolate #1	206	γ
Saratoga	195	γ
Saratoga	100	γ
Saratoga	196	γ
070	103	γ
070	193	γ
070	194	γ

*6 day cultures

Table 7. Spectral patterns of the five day old nontoxic strains closely resembling Clostridium botulinum, type E

Strain	Spectrum #	Spectral pattern
GB-3	221*	β
GB-3	222*	β
15Ati	233*	β
15Ati	234*	β
805Bb	124	γ
805Bb	223*	γ
805Bb	224*	γ
15Cbti	213	γ
15Cbti	214	γ
15Cbti	75*	γ
15Cbti	76*	γ
34-1	217	γ
34-1	218	γ
170C	232	γ
170C	231	γ
170IX	216	γ
170IX	215	γ
8Ati	219*	γ
8Ati	220*	γ
S-9	235	γ
S-9	236	γ
900D	237	γ
900D	238	γ
	Variable spectra	
066BNT	125	γ
066BNT	180	λ
066BNT	181	λ

*Cultures #221, 222, 219, 220 were 6 days old; cultures #233, 234, 223, 224, 75 and 76 were 7 days old

Table 8. Spectral patterns of the five day old toxic strains of Clostridium botulinum, types A B, and F

Strain	Spectrum #	Spectral pattern
Type F	257	γ
Type F	258	γ
Type F	128	γ
Type A		
5A	249	δ
5A	250	δ
5A	134	δ
5A	135	δ
33A	136	δ
33A	137	δ
33A	252	δ
33A	251	δ
Type B		
113B	254	δ
113B	253	δ
113B	130	δ
115B	129	δ
115B	256	δ
115B	255	δ

Table 9. Spectral patterns of the five day old miscellaneous Clostridia

Strain	Spectrum #	Spectral pattern
Cl. tetani	297	γ (2 spectra)
Cl. tetani	282	γ (2 spectra)
Cl. difficile	306	γ (2 spectra)
Cl. difficile	285	γ (2 spectra)
Cl. butyricum	281	γ (2 spectra)
Cl. butyricum	305	γ (2 spectra)
Cl. novyi	290	γ (2 spectra)
Cl. novyi	311	γ (2 spectra)
Cl. fallax	284	δ (2 spectra)
Cl. fallax	308	δ (2 spectra)
Cl. tertium	283	δ (2 spectra)
Cl. tertium	307	δ (2 spectra)
Cl. welchii	261	δ
Cl. welchii	309	δ (2 spectra)
Cl. welchii	260	δ
Cl. welchii	286	δ (2 spectra)
Cl. histolyticum	279	δ (2 spectra)
Cl. histolyticum	303	δ (2 spectra)
Cl. sporogenes	302	δ (2 spectra)
Cl. sporogenes	132	δ
Cl. sporogenes	280	δ (2 spectra)
Cl. sporogenes	259	δ
Cl. sporogenes	131	δ
	Variable spectra	
Cl. septicum	291	γ (2 spectra)
Cl. septicum	262	δ

the two and five day age levels is given in Tables 1-9. It can be seen from these tables that the great bulk of the clostridia fall in patterns α, β, γ, or δ. The other organisms examined seem to give their own patterns. The pattern given by an organism can show some variance with age. The type β pattern does not seem to be as subject to age variation as other patterns.

The Effect of Age of Agar Cultures on the Spectra

When comparing the overall percentage distribution of the two day and the five day old cultures, the first thing noticed is that the overall variability is about 22%, in the two day old cultures, but only 2.5% in the five day old cultures. By this observation, one out of every five strains of the two day cultures, but only one out of 40 of the five day cultures examined will show variability. Almost all of the spectra examined could be classified into four basic patterns. They are types α, β, γ, and δ.

It is very interesting to note that the type α spectral pattern has completely disappeared at the five day age, although it was present as the basic spectral pattern of 51.2% of the two day old cultures examined.

The significance of this is immediately seen if the experiment in Figure 18 (depicting the effect of age on the spectral pattern of

cultures) is examined. Looking at the 44 hr. old culture, it is seen to be characterized by a peak at 7.2 μ, and a peak at 8.1 μ. As the culture ages, the 7.2 μ peak gradually deepens, the peak at 8.1 μ gradually fades, and a new peak at 8.7 μ gradually begins to form.

The pattern of the 44 hr. old culture is almost identical to spectral pattern α, but as the culture ages, the pattern changes, and the 144, 168, and 186 hr. old cultures are seen to be almost identical to spectral pattern γ. Thus the disappearance of pattern α and the increase in pattern γ is seen to be associated with a variation in the spectral pattern due to age. This change may be partially associated with the accumulation of metabolic byproducts, such as glycogen.

Spectral pattern type β (Figure 10) is known to be associated with the accumulation of large amounts of glycogen by the cell (Levine, 1953). In the two day old cultures, this pattern is seen exclusively in three strains of Clostridium botulinum, type E. They are VH#1, VH#2, and Beluga. These same strains continue to exhibit the strong type β pattern at the five day age, while all of the other toxic type E strains revert from the type α pattern to the type γ pattern. (See Tables 23 and 24, Figures 9 and 11.) Meanwhile, the type β pattern appears in the GB-3, and 15Ati strains of nontoxic Cl. botulinum type E, at the five day age. Only 15Ati gave a 50% occurrence of the type β pattern at the two day age (the other 50% was type α pattern), while GB-3 was exclusively type γ at the two day age level. Meanwhile, the rest of the

nontoxic type E strains reverted from a combination of type α and type γ spectral patterns at the two day age to pattern γ exclusively at the five day age.

The type β spectral pattern is seen twice out of four examinations in Cl. butyricum, and once out of three examinations in Cl. septicum, but it disappears in these organisms at the five day age. The ε and λ patterns (which are seen to be very similar) are seen once out of three examinations in two day cultures of Cl. bifermentans and twice out of eight times in an examination of the two day old cultures of Cl. sporogenes. The type λ spectral pattern is also seen twice in Cl. botulinum Type E Strain 066BNT (five day old culture). They are never seen again, and are very unusual. The significance of these two types of patterns (ε and λ) is not immediately obvious. Virtually all of the spectral patterns of the two day old miscellaneous clostridia are of type α. In the five day old spectra, patterns α and β disappear in the miscellaneous clostridia, and the spectral patterns of the organisms become almost equally divided between patterns γ and δ (Tables 4 and 9).

An examination of Figures 19-24 indicates that there are no great differences between Cl. botulinum Type E Toxic, Nontoxic "E-like" clostridia or the miscellaneous clostridia, in the region from 6.0 μ to 8.0 μ, at either the two or the five day age (other than those which have been described above).

Figure 18. The effect of culture age on the IR absorption spectrum of a non-toxic "E-like" Clostridium (strain 170C)

Cultures grown on TPGY agar, dried one day in an evacuated desiccator over P_2O_5.

Notice the gradual deepening of the peak at 7.2 μ.

Notice the gradual fading of the peak at 8.1 μ.

Notice the gradual formation of the peak at 8.7 μ.

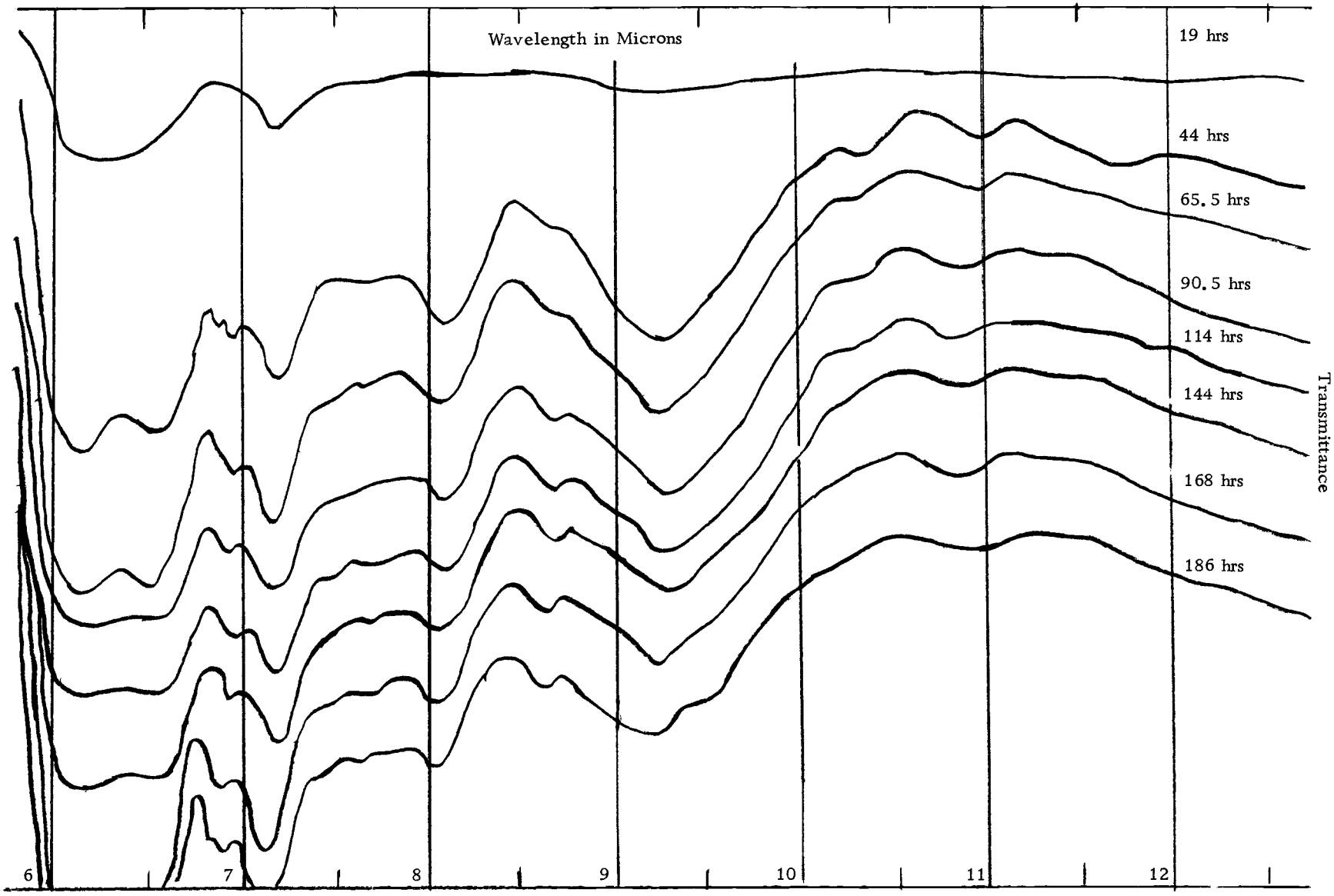

Wavelength in Microns
19 hrs
44 hrs
65.5 hrs
90.5 hrs
114 hrs
144 hrs
168 hrs
186 hrs
Transmittance
6
7
8
9
10
11
12

Figure 19. Cl. botulinum, nontoxic "E-like" strains.

Two day old cultures on TPGY agar

Sample #	Strain	Sample #	Strain
165	170IX	172	170C
166	34-1	176	900D
170	15Ati	179	GB-3

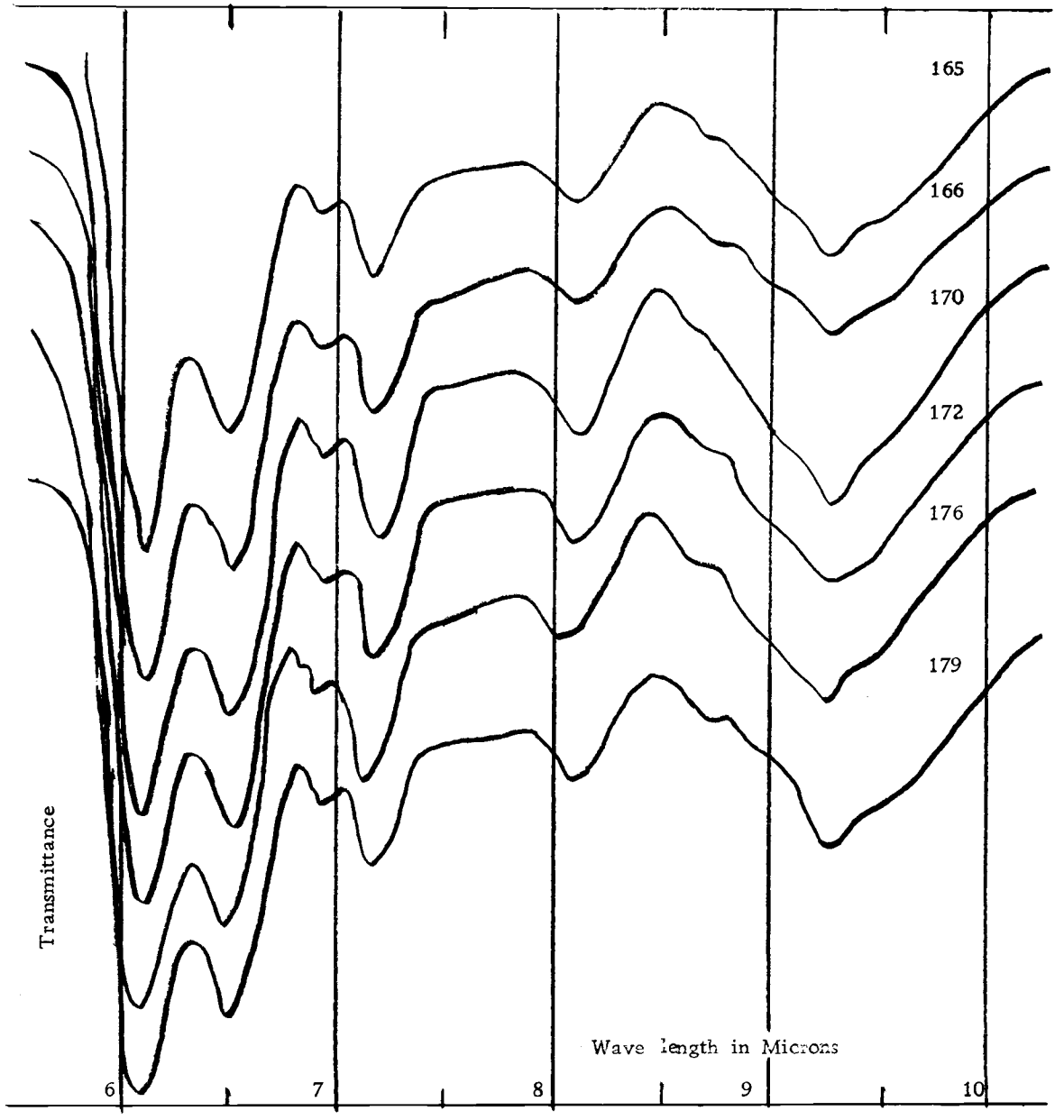

165
166
170
172
176
179
Transmittance
Wave length in Microns
6
7
8
9
10

Figure 20. Cl. botulinum, toxic type E strains.

Two day old cultures on TPGY agar

Sample #	Strain	Sample #	Strain
152	170	140	Iwahai
147	Tuna Can	151	VH#1
146	Saratoga	142	Beluga

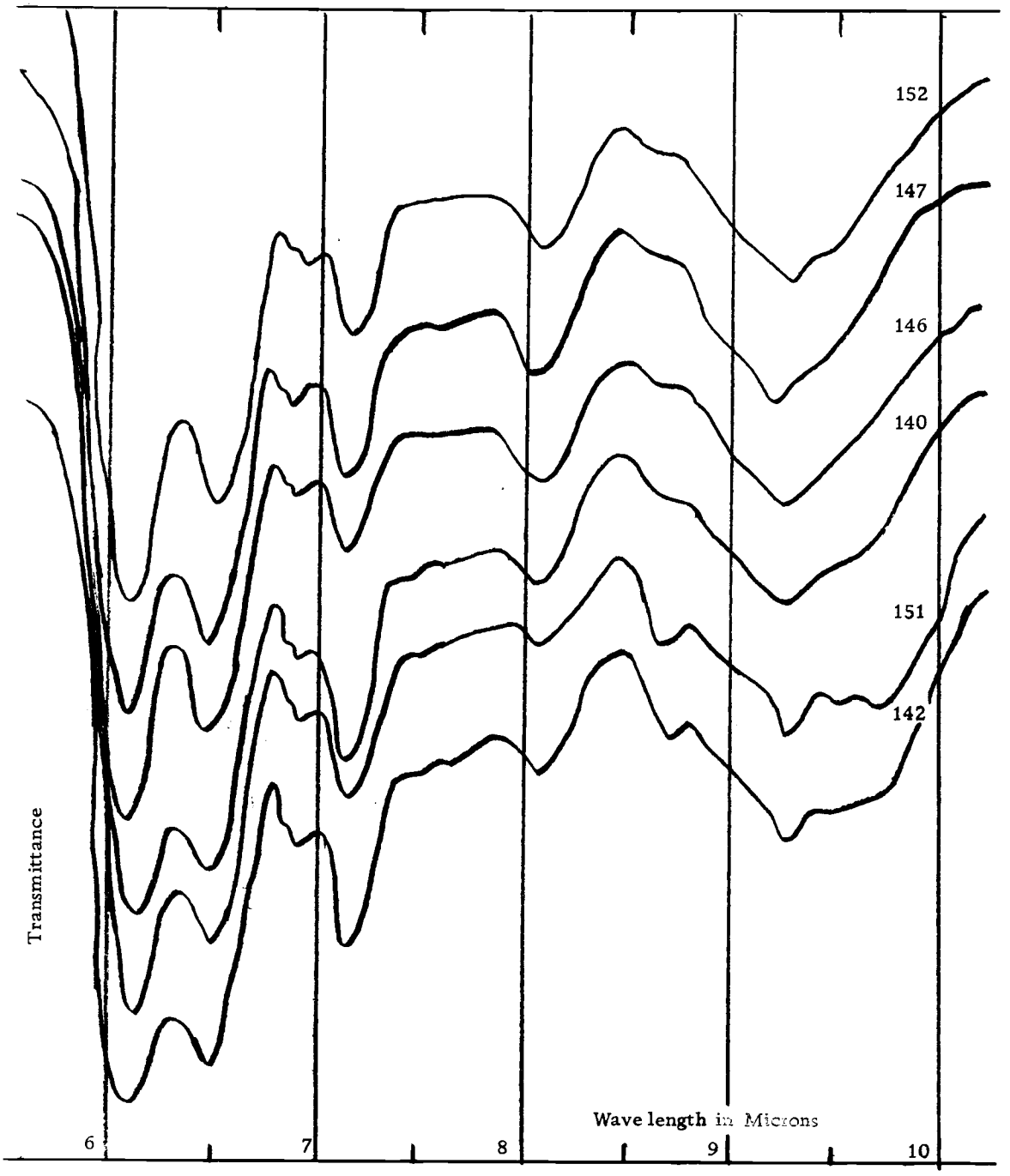

152
147
146
140
151
142
Transmittance
Wave length in Microns
6
7
8
9
10

Figure 21. Miscellaneous Clostridia, two day old cultures on TPGY Agar

Sample #	Strain	Sample #	Strain
272	Cl. histolyticum	297	Cl. tetani
269	Cl. sporogenes	299	Cl. fallax
275	Cl. tertium	273	Cl. butyricum

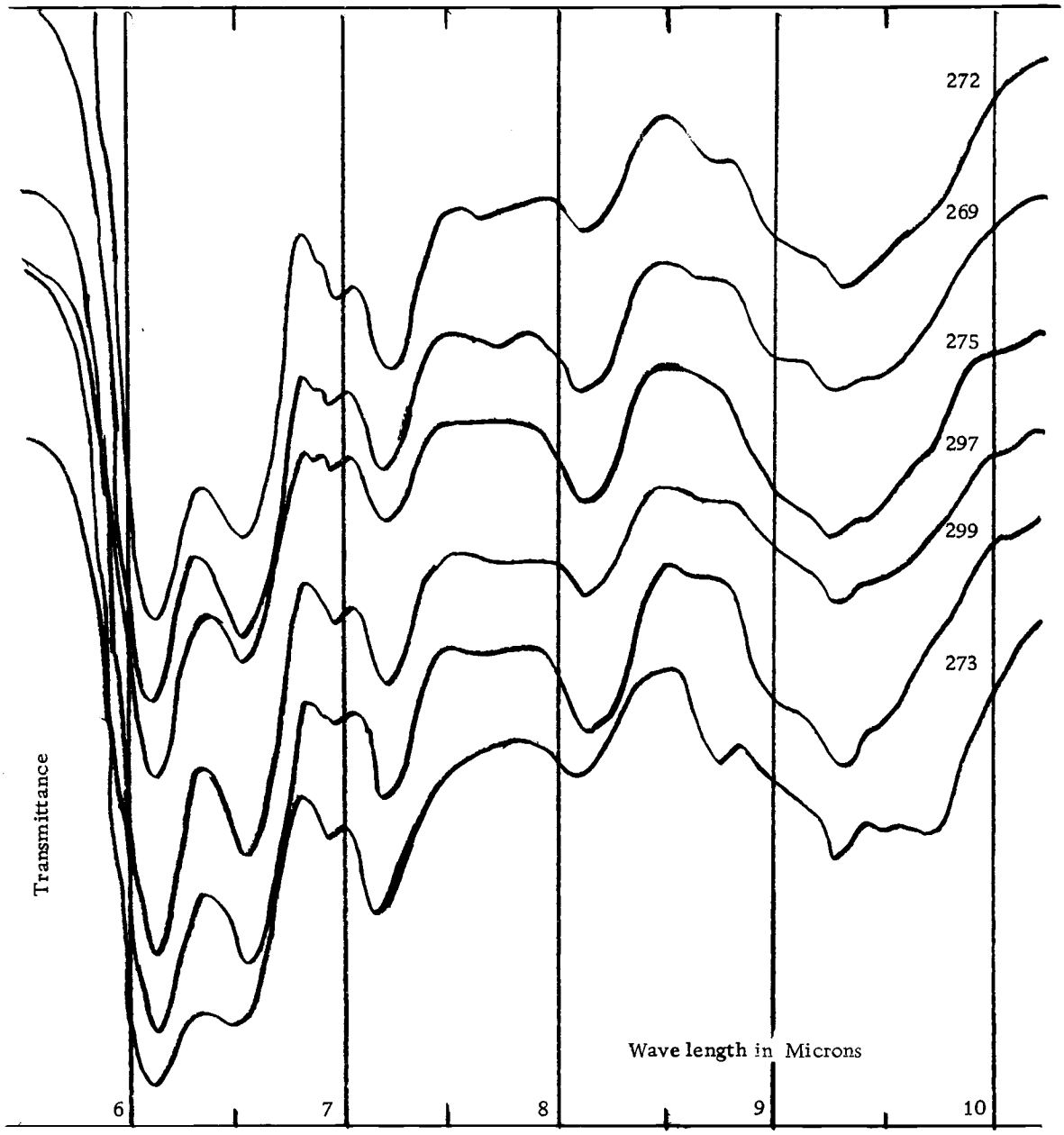

272
269
275
297
299
273
Transmittance
Wave length in Microns
6
7
8
9
10

Figure 22. Cl. botulinum, nontoxic 'E-like' strains

Five day old cultures on TPGY agar

Sample #	Strain	Sample #	Strain
213	15Cbti	217	34-1
219	8Ati	215	170IX
223	805Bb	221	GB-3

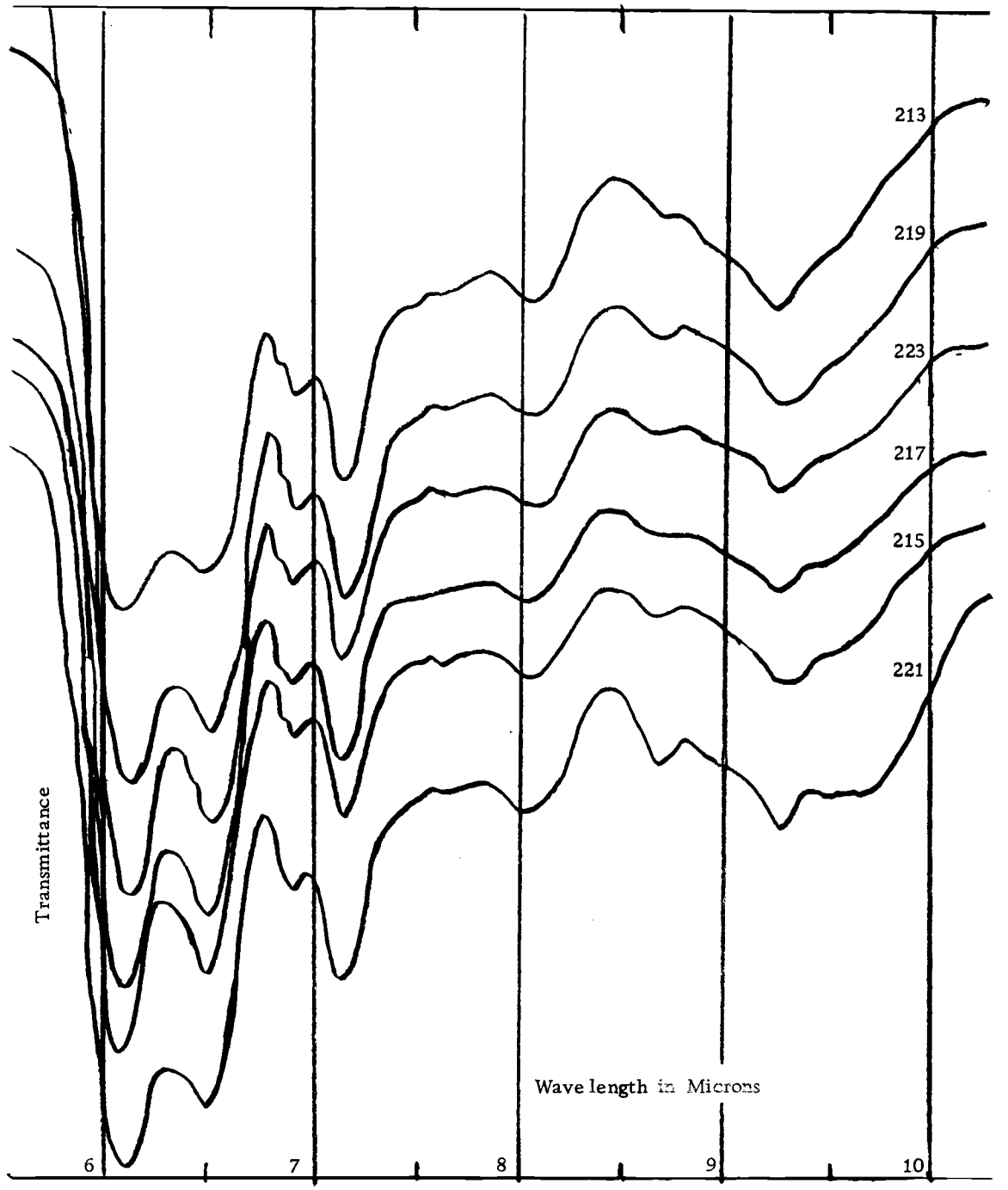

213
219
223
217
215
221
Transmittance
Wave length in Microns
6
7
8
9
10

Figure 23. Cl. botulinum, toxic type E strains

Five day old cultures on TPGY agar

Sample #	Strain	Sample #	Strain
195	Saratoga	109	Iwahai
191	Tuna Can	108	Alaska
190	Kalamazoo	188	VH#1

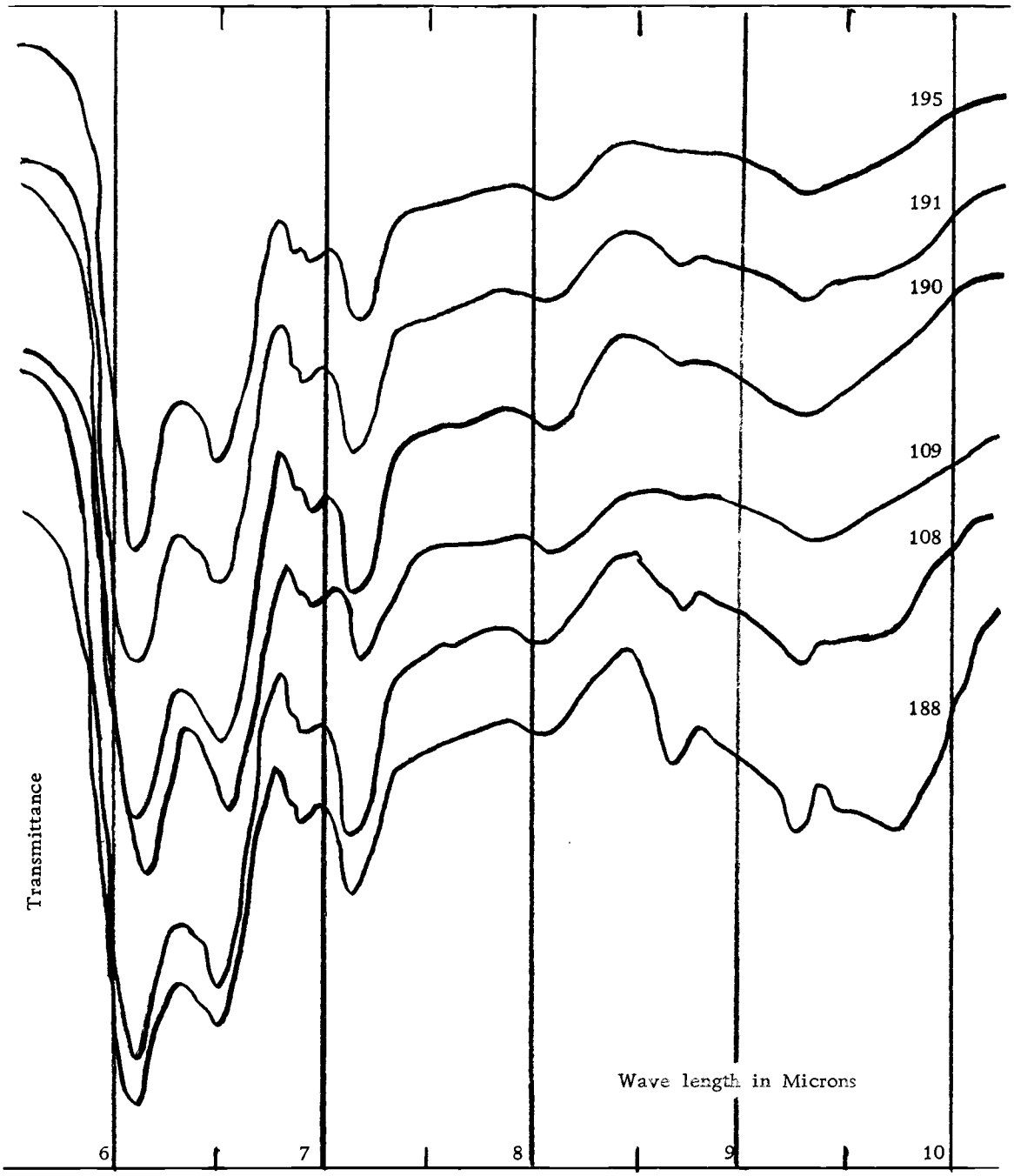
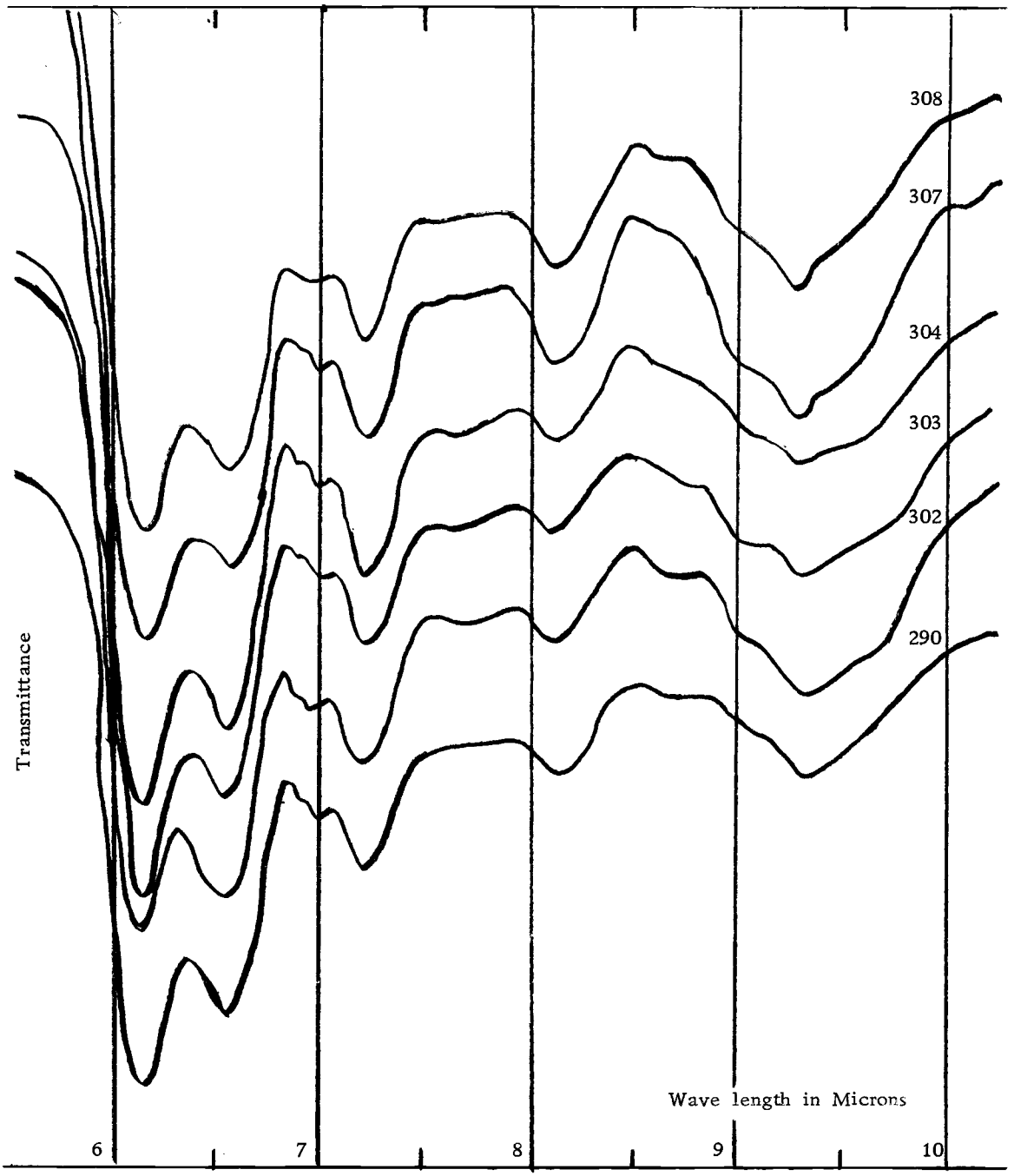

195
191
190
109
108
188
Transmittance
Wave length in Microns
6
7
8
9
10

Figure 24. Miscellaneous Clostridia, five day old cultures on TPGY agar

Sample #	Strain	Sample #	Strain
308	Cl. fallax	303	Cl. histolyticum
307	Cl. tertium	302	Cl. sporogenes
304	Cl. tetani	290	Cl. novyi


308
307
304
303
302
290
Transmittance
Wave length in Microns
6
7
8
9
10

Although the cultures noted in footnotes 3, 4, and 5 were older than the rest of those in their respective tables, the data did not seem to be altered by their presence.

Table 10. The distribution of spectral patterns as a function of age. The two day old toxic type E strains of Cl. botulinum

Total number of strains examined: 14
Number of strains represented by more than one spectral pattern: 1 or 7.2%
Iwanai: one type β pattern, two type α patterns
Number of strains of spectral type α :10 or 71.5%
Number of strains of spectral type β : 3 or 21.4%

Table 11. The distribution of spectral patterns as a function of age. The two day old nontoxic "E-like" strains of Cl. botulinum

Total number of strains examined: 11
Number of strains represented by more than one spectral pattern: 4 or 36.4%
900D: two type α patterns, two type γ patterns
15Ati: two type α patterns, two type γ patterns
805Bb: two type α patterns, two type γ patterns
170IX: one type α pattern, two type γ patterns
Number of strains of spectral type α: 4 or 36.4%
Number of strains of spectral type γ: 3 or 27.1%

Table 12. The distribution of spectral patterns as a function of age. The two day old type A strains of Cl. botulinum

Total number of strains examined: 2
Number of strains represented by more than one spectral pattern: 0
Number of strains of spectral type δ: 2

Table 13. The distribution of spectral patterns as a function of age. The two day old type B strains of Cl. botulinum

Total number of strains examined: 2

Number of strains represented by more than one spectral pattern: 0

Number of strains of spectral type δ : 2

Table 14. The distribution of spectral patterns as a function of age. The two day old type F strains of Cl. botulinum

Total number of strains examined: 1

Number of strains represented by more than one spectral pattern: 0

Number of strains of spectral type γ: 1

Table 15. The distribution of spectral patterns as a function of age. The two day old miscellaneous clostridia

Total number of strains examined: 11

Number of strains represented by more than one spectral pattern: 4 or 36.4%

Cl. septicum: two type α patterns, one type β pattern

Cl. butyricum: two type α patterns, two type β patterns

Cl. bifermentans: Two type α patterns, one type ϵ pattern

Cl. sporogenes: six type α patterns, two type λ patterns

Number of strains of spectral type α : 7 or 63.5%

Table 16. The distribution of spectral patterns as a function of age. The two day old miscellaneous bacteria

Total number of strains examined: 6
Number of strains represented by more than one spectral pattern: 1 or 16.7%
Bacillus megatherium: two type G patterns, two type H patterns
Number of strains of spectral type α: 2 or 33.3%
Number of strains of spectral type I: 1 or 16.7%
Number of strains of spectral type J: 1 or 16.7%
Number of strains of spectral type G: 1 or 16.7%

Table 17. The distribution of spectral patterns as a function of age. The five day old type E toxic strains of Cl. botulinum

Total number of strains examined: 14
Number of strains represented by more than one spectral pattern: 0
Number of strains of spectral type β : 3 or 21.5%
Number of strains of spectral type γ : 11 or 78.7%

The same strains which consistently show type β spectra at the two day age also show this at the five day age (compare tables 1 and 6). All of the rest of the strains revert from the type α spectra at two days to the type γ spectra at five days. The difference between the type α and γ spectra seems to be in the formation of a peak at 8.7 μ. The development of this peak with age is nicely brought out in a study of the effect of age on the spectral patterns. (See Figure 18.)

It can be suspected that this peak is due to the storage of small amounts of glycogen.

Table 18. The distribution of spectral patterns as a function of age. The five day old nontoxic "E-like" strains of Cl. botulinum

Total number of strains examined: 11
Number of strains represented by more than one spectral pattern: 1 or 9.1%
Number of strains of spectral type β : 2 or 18.2%
Number of strains of spectral type γ : 8 or 72.7%

Table 19. The distribution of spectral patterns as a function of age. The five day old type A strains of Cl. botulinum

Total number of strains examined: 2
Number of strains represented by more than one spectral pattern: 0
Number of strains of spectral type δ: 2

Table 20. The distribution of spectral patterns as a function of age. The five day old type B strains of Cl. botulinum

Total number of strains examined: 2
Number of strains represented by more than one spectral pattern: 0
Number of strains of spectral type δ: 2

Table 21. The distribution of spectral patterns as a function of age. The five day old type F strains of Cl. botulinum

Total number of strains examined: 1
Number of strains represented by more than one spectral pattern: 0
Number of strains of spectral type γ: 1

Type F was consistently spectral type γ at both the two and the five day ages (compare Tables 14 and 21). Types 5A, 33A, 113B, and 115B were consistently spectral type δ at both the two and the five day ages (compare tables 12, 13, 19, and 20).

Table 22. The distribution of spectral patterns as a function of age. The five day old miscellaneous clostridia

Total number of strains examined: 10
Number of strains represented by more than one spectral pattern: 1 or 10%
Cl. septicum: two type γ patterns, one type δ pattern
Number of strains of spectral type γ: 4 or 40%
Number of strains of spectral type δ: 5 or 50%

Looking at the two day old miscellaneous clostridia (Table 4), it is seen that just about all of the spectral patterns are of type α. The type β glycogen pattern and the type α pattern were both observed with Cl. septicum, and Cl. butyricum. The type ε and λ spectral patterns (seen in Cl. bifermentans and Cl. sporogenes) are

very similar to each other and very different from all other spectral types. This pattern is also seen once in Cl. botulinum type E, Strain 066BNT (Table 7). This seems to be a rarely seen and variant pattern. The physiological significance of this pattern is not immediately apparent, but it is certainly not consistent with any of the other patterns seen.

In the five day old cultures (Table 9), Cl. tetani, Cl. difficile, Cl. butyricum, and Cl. novyi seem to have reverted from the type α spectral pattern to the type γ spectral pattern. This pattern is associated with the formation of a peak at 8.7 μ (which is thought to be associated with glycogen formation). Cl. fallax and Cl. tertium have reverted to the type δ pattern, as have Cl. histolyticum and Cl. sporogenes.

Table 23. Spectral pattern distributions of the two day old cultures

Patterns	α Strains	β Strains	γ Strains	δ Strains	Variable Strains
Two day cultures Overall	21	3	4	4	9
Cl. botulinum Toxic type E	10	3			1
Cl. botulinum Nontoxic "E-like"	4		3		4
Cl. botulinum Type A				2	
Cl. botulinum Type B				2	
Cl. botulinum Type F			1		
Miscellaneous Clostridia	7				4

Table 24. Spectral pattern distribution of the five day old cultures

Patterns	α Strains	β Strains	γ Strains	δ Strains	Variable Strains
Five day cultures Overall		5	25	9	1
Cl. botulinum Toxic type E		3	11		
Cl. botulinum Nontoxic "E- like"		2	8		1
Cl. botulinum Type A				2	
Cl. botulinum Type B				2	
Cl. botulinum Type F			1		
Miscellaneous Clostridia			4	5	1

Serious Limitations of IR Spectra as an Aid in Identification of Clostridial Species

Using Two Day Old Agar Cultures

All clostridia examined could be classified in one of six spectral patterns, shown in Figures 9 to 13. The spectral classification of each of these organisms is shown in Tables 1, 2, 3, and 4. It is readily apparent that there is little relationship between species and spectral classification. Most of the pathogenic clostridia could not be distinguished from the non pathogens by this means. Neither is it

possible to distinguish a toxigenic strain of Cl. botulinum type E from nontoxigenic "E-like" strains. The limited data suggest, however, that given a culture of Cl. botulinum of unknown type, the IR spectrum of a two day culture on TPGY might give some information as to the probable type. Thus an α or β spectrum would suggest a type E culture, a δ spectrum a type A or B culture, and a type γ spectrum a type F culture. Data on a larger number of strains of each immunological type would be needed to warrant even these limited inferences; and of course, immunological confirmation of type would still be needed.

The data in Table 5 and Figures 14 to 17 suggest that the IR spectra of Sarcina lutea, Staphylococcus aureus, and Bacillus subtilis are different from any of those observed with the clostridia, but the observation is limited to one strain of each organism.

Using Five Day Old Agar Cultures

When these somewhat older cultures on TPGY agar were examined, it was found that each clostridial strain could be classified in one of four spectral patterns, instead of six, as observed with the two day cultures. These patterns were essentially the same as four of those found with the younger cultures, i.e., β, γ, δ, and ε. The patterns found with each of the clostridia examined are shown in Tables 6, 7, 8, and 9.

Again, the inferences that could be drawn from the spectrum of an unknown clostridial culture are extremely limited. Given a strain of toxigenic Cl. botulinum of unknown immunological type, a β spectrum would suggest a type E culture, a γ spectrum a type E or F culture, and a δ spectrum a type A or B culture. With the five day cultures also, pathogenic and toxin producing clostridia could not be distinguished from other non pathogenic species.

The Effect of Washing on the IR Spectra of Some Clostridial Cultures

The spectral patterns of washed broth cultures of clostridia are variations of spectral patterns β and δ, and hence are called ββ and δδ. (See Figures 25 and 26.) The variation is found in the region from 7.5 μ to 8.5 μ. In an unwashed broth culture, the region from 7.5 μ to 8.0 μ slopes upward. In a washed broth culture, this section of the curve slopes downward. This is because the entire region of the spectrum from 6.2 μ to 8.2 μ is raised or considerably decreased in intensity from the rest of the spectral curve. (See Figures 27-29.)

This region from 6.2 μ to 8.1 μ is the region in which the bonds found in proteins have their characteristic absorption peaks. The peak at 6.6 μ is typical of CONH and N-H bonds, while the peak at 6.1 μ results partially from the C=O bond (Norris, 1959). Another peak is noted at about 7.2 μ. This is probably due to the carboxyl group (Bellamy, 1958, p. 224).

The unwashed broth cultures of 066B and 066BNT both remained type α as in their agar cultures; however, after washing, they were both found to be spectral type δδ. Strain 15Ati was found to vary between the α and β spectral types on agar. (See Table 25.) As

Figure 25. Spectral pattern ββ (washed broth culture)

Spectral #	Organism		Age
	Cl. botulinum Type E strains		
#329	VH#2	(toxic)	2 day
#327	VH#1	(toxic)	2 day
#351	Saratoga	(toxic)	2 day
#350	Beluga	(toxic)	2 day

Cultures #327 and #329 were grown in TPGY broth. Cultures #350 and #351 were grown in TPG biphasic culture. All cultures were grown at 28°C. They were washed twice with distilled water.

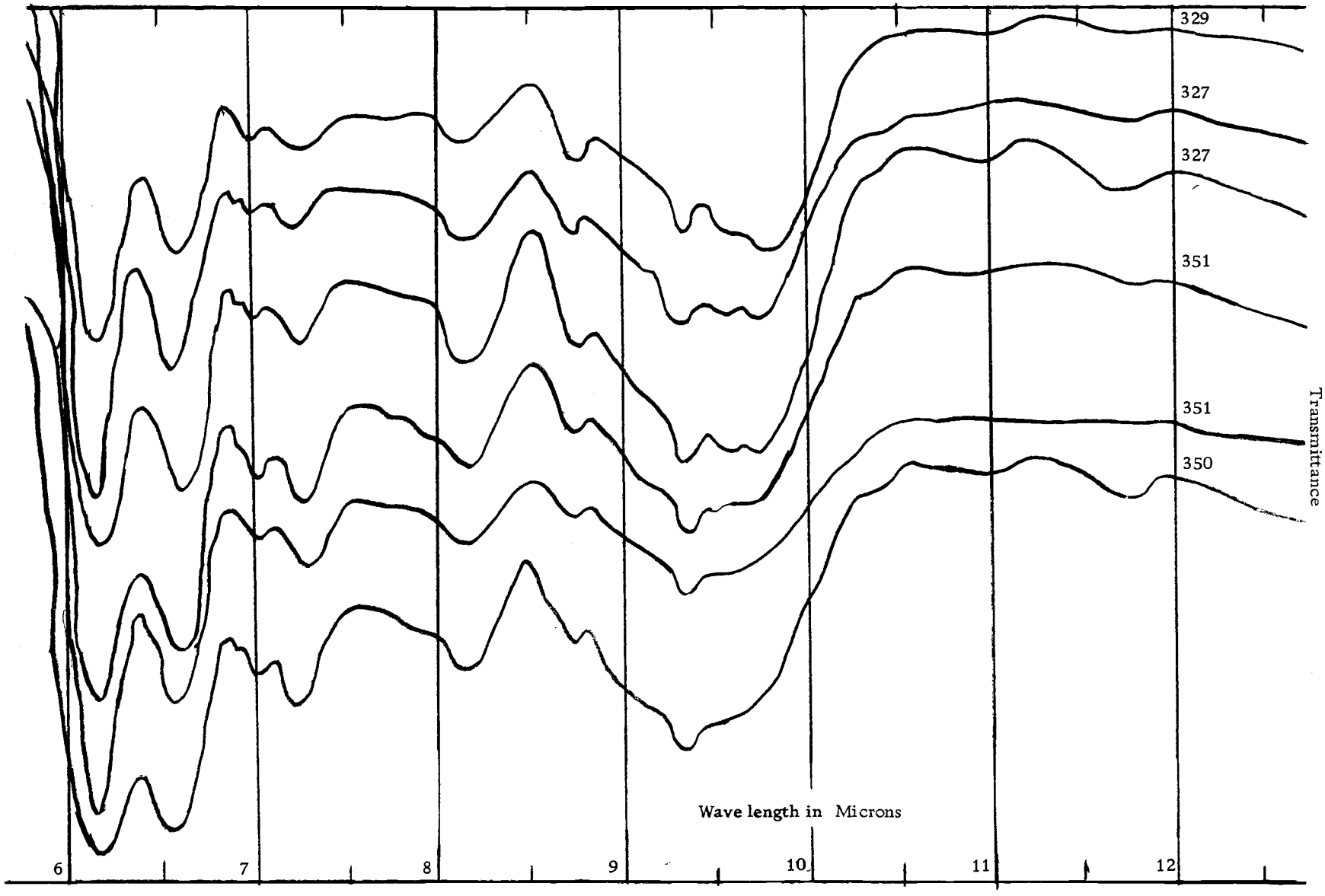
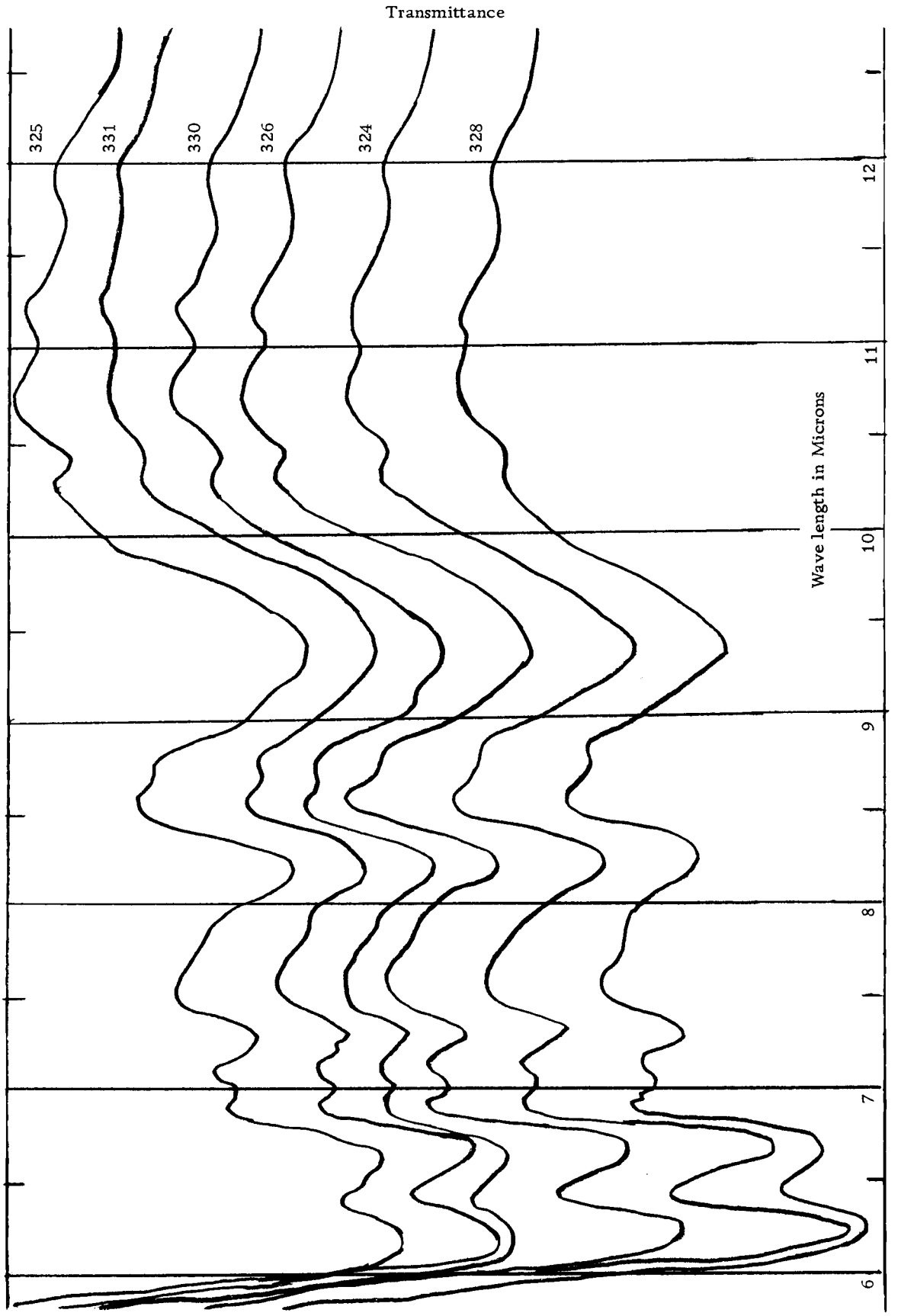

329
327
327
351
351
350
Transmittance
Wave length in Microns
6
7
8
9
10
11
12

Figure 26. Spectral pattern δδ (washed broth culture)

Spectral #	Organism		Age
	Cl. botulinum "E-like"		
#325	066BNT	(nontoxic)	2 day
#330	34-1	(nontoxic)	2 day
#326	15Ati	(nontoxic)	2 day
#324	066BNT	(nontoxic)	2 day
#331	Cl. butyricum		2 day
#328	Cl. difficile		2 day

All cultures were grown in TPGY broth at 28°C. They were washed twice with distilled water.


Transmittance
325
331
330
326
324
328
6
7
8
9
10
11
12
Wave length in Microns

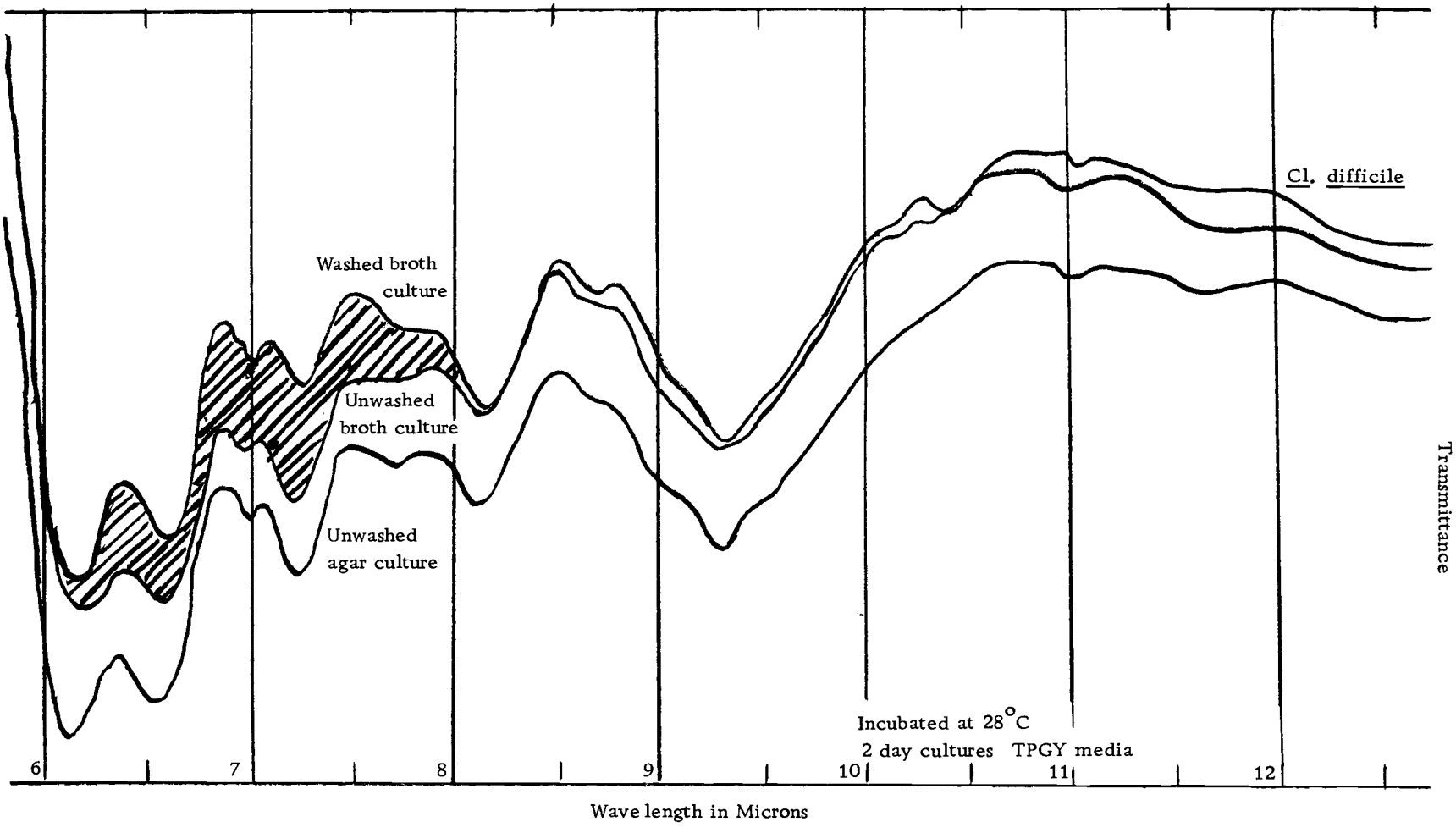


Figure 27. A comparison of the spectra of washed and unwashed cultures

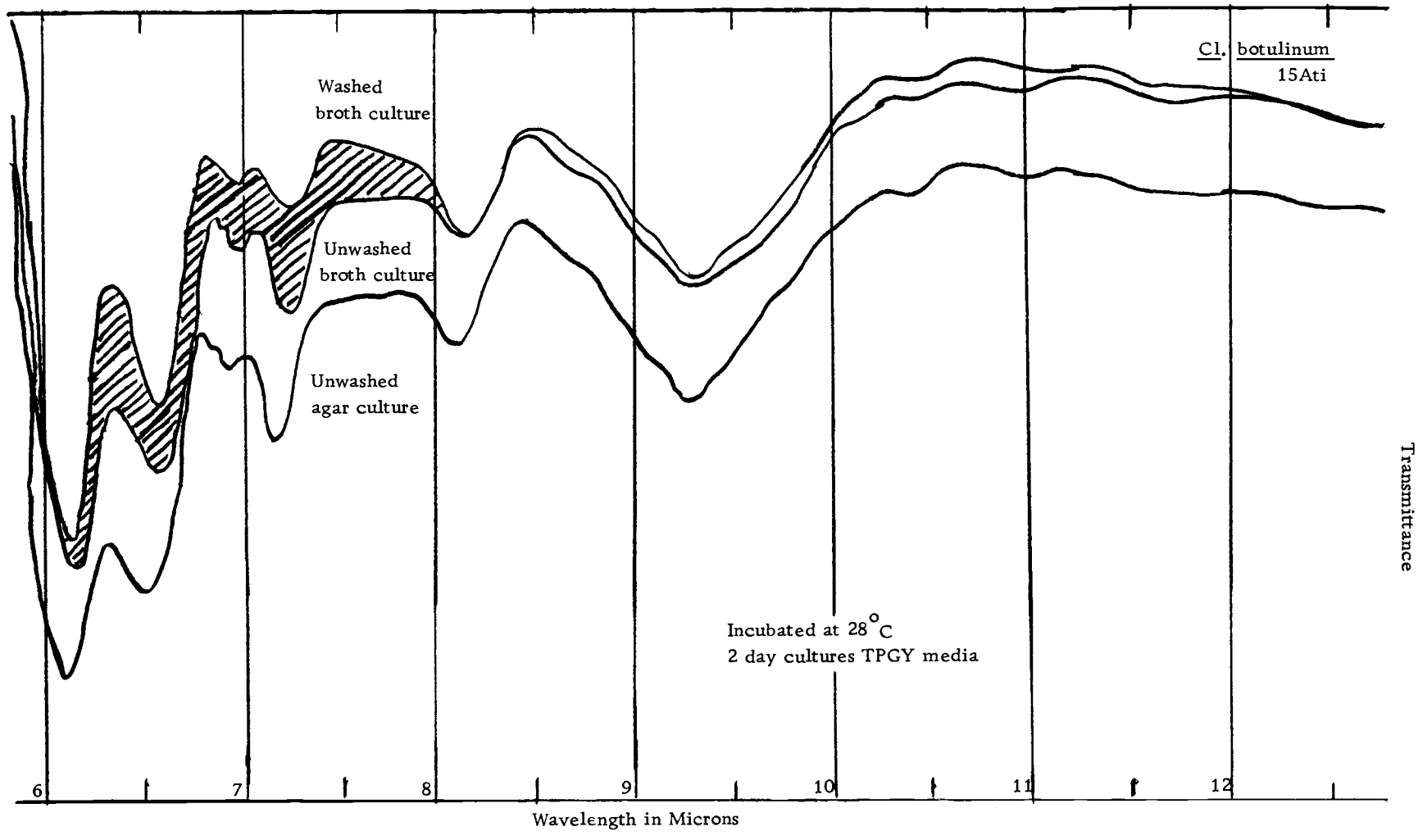


Figure 28. A comparison of the spectra of washed and unwashed cultures

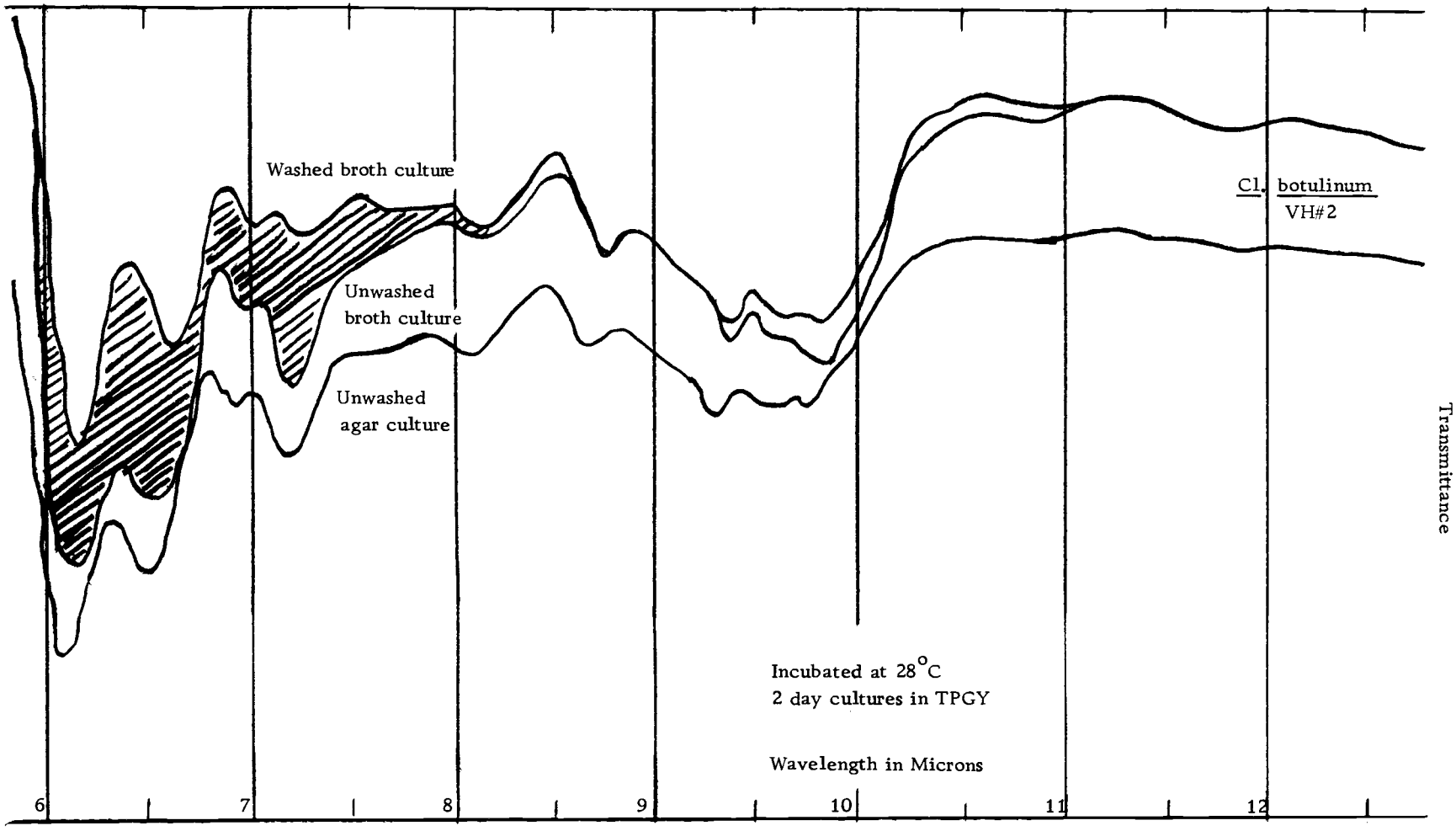


Figure 29. A comparison of the spectra of washed and unwashed cultures

unwashed two day broth cultures, they were found as type α, but after washing, they exhibited spectral type δδ. The two day old agar cultures of Cl. butyricum were spectral type α, although pattern β was seen once. In broth culture, it remained type α, but after being washed, it reverted to type δδ.

The cultures VH#1 and VH#2 were of spectral type β in both two day agar cultures, and two day broth cultures. After washing, it was found that they were spectral type ββ. In two day old agar cultures, Cl. difficile was found to be spectral type α, and Cl. sporogenes was found to be usually type α, but also type λ. In broth culture, Cl. sporogenes and Cl. difficile were type δ, and upon being washed, reverted to type δδ.

It is very interesting to note that all cultures except VH#1 and VH#2 reverted to spectral pattern δδ in the washed broth culture state. (See Table 25.)

This change from pattern α to pattern δδ in washed broth cultures shows a change in the spectral region from 8.5 μ to 10.5 μ after washing. Since this region is dominated by nucleic acids, and polysaccharides, it may be that the washing process somehow alters the polysaccharide composition on the exterior of the cell.

It is also noted that there is a large general decrease in the absorption of the region from 6.1 μ to 8.1 μ in the washed broth cultures. This holds true in every case examined. As a measure of

Table 25. A comparison of the spectral patterns of the two day agar cultures, broth cultures, and washed broth cultures

Organism	Spectral #	Spectral type	Culture type
Cl. botulinum			
Strains			
066B	80	α	Agar
066B	149	α	Agar
066B	148	α	Agar
066B	318	α	Broth
066B	325	δδ	Washed broth
VH#1	151	β	Agar
VH#1	150	β	Agar
VH#1	79	β	Agar
VH#1	320	β	Broth
VH#1	327	ββ	Washed broth
VH#2	157	β	Agar
VH#2	156	β	Agar
VH#2	90	β	Agar
VH#2	321	β	Broth
VH#2	329	ββ	Washed broth
066BNT	169	α	Agar
066BNT	168	α	Agar
066BNT	317	α	Broth
066BNT	324	δδ	Washed broth
15Ati	171	α	Agar
15Ati	170	α	Agar
15Ati	60	β	Agar
15Ati	61	β	Agar
15Ati	319	α	Broth
15Ati	326	δδ	Washed broth
34-1	167	α	Agar
34-1	166	α	Agar
34-1	332	α	Broth
34-1	330	δδ	Washed broth
Cl. butyricum	93	α	Agar
Cl. butyricum	94	α	Agar
Cl. butyricum	273	β	Agar
Cl. butyricum	333	α	Broth
Cl. butyricum	331	δδ	Washed broth
Cl. difficile	278	α	Agar
Cl. difficile	335	δ	Broth
Cl. difficile	328	δδ	Washed broth
Cl. sporogenes	77	λ	Agar
Cl. sporogenes	78	λ	Agar
Cl. sporogenes	112	α	Agar
Cl. sporogenes	113	α	Agar
Cl. sporogenes	269	α	Agar
Cl. sporogenes	274	α	Agar
Cl. sporogenes	314*	δ	Broth
Cl. sporogenes	316	δ	Broth
Cl. sporogenes	322	δ	Broth
Cl. sporogenes	334	δδ	Washed broth

*4 day old culture

the degree of "thinning" of the spectra in this region, the ratio of the OD at 7.2 μ/OD at 8.1 μ was adopted. A comparison of the average of these ratios for two day old agar cultures, broth cultures, and washed broth cultures of the same series of organisms and strains is given below. (Also see Table 27.)

Table 26. Ratio of OD at 7.2 μ to OD at 8.1 μ

Agar cultures	Broth cultures	Washed broth cultures
1.601	1.679	0.9024

The large change seen in the broth cultures which have been washed is in the area of the spectrum associated with the types of bonds found in proteins. Apparently proteins or amino acids or polypeptides are removed from the outside of the cell by the washing process.

Table 27. The variation of the OD 7.2 μ/OD 8.1μ ratio as a function of cell washing in the Clostridia. W = washed, U = unwashed, A = agar, B = broth

Organism	Spectrum #	Agar or broth washed or unwashed	7.2 μ OD / 8.1 μ OD
Cl. botulinum Strains			
VH#1	151	U-A	1.74
VH#1	150	U-A	1.79
VH#1	79	U-A	1.6
VH#2	156	U-A	1.62
VH#2	90	U-A	2.0
VH#2	157	U-A	1.57
066BNT	169	U-A	1.41
066BNT	168	U-A	1.68
15Ati	61	U-A	1.83
15Ati	60	U-A	1.87
15Ati	171	U-A	1.38
15Ati	170	U-A	1.46
34-1	167	U-A	1.4
34-1	166	U-A	1.62
066B	80	U-A	1.25
066B	149	U-A	1.67
066B	148	U-A	1.72
Cl. difficile	278	U-A	1.21
Cl. butyricum	273	U-A	1.56
Cl. butyricum	273	U-A	1.67
Cl. sporogenes	78	U-A	1.89
Cl. sporogenes	77	U-A	1.87
Cl. sporogenes	113	U-A	1.8
Cl. sporogenes	112	U-A	1.79
Cl. sporogenes	269	U-A	1.28
Cl. sporogenes	269	U-A	1.31
Cl. sporogenes	274	U-A	1.46
Cl. sporogenes	274	U-A	1.4
Cl. butyricum	333	U-B	1.51
Cl. botulinum Strains			
15Ati	319	U-B	1.58
066B	318	U-B	1.53
066BNT	317	U-B	1.84
VH#1	320	U-B	1.76
VH#2	321	U-B	1.81
Cl. sporogenes	322	U-B	1.93
Cl. sporogenes	316	U-B	1.83
Cl. sporogenes	314 - 4 days	U-B	1.77

(Continued)

Table 27. Continued

Organism	Spectrum #	Agar or broth washed or unwashed	7.2 μ OD / 8.2 μ OD
Cl. hemolyticum	323	U-B	1.45
Cl. hemolyticum	315*	U-B	2.0
Cl. difficile	335	U-B	1.38
Cl. botulinum Strains			
VH#1	327	W-B	0.95
VH#2	329	W-B	1.04
066B	325	W-B	0.78
066B	325	W-B	0.88
34-1	330	W-B	0.80
34-1	330	W-B	0.85
15Ati	326	W-B	0.87
15Ati	326	W-B	0.88
066BNT	324	W-B	0.93
066BNT	324	W-B	0.88
Cl. butyricum	331	W-B	0.87
Cl. butyricum	331	W-B	0.94
Cl. difficile	328	W-B	0.95
Cl. difficile	328	W-B	0.89

*4 days

Comparison of the IR Spectra of Spores and Vegetative Cells of Clostridial Cultures

TPGY is a very rich medium and essentially no spores were seen in cultures grown in this medium. Thus the biphasic technique was used to obtain sporulation. All cultures were washed in distilled water before recording spectra. Cl. botulinum Type E, Strains VH#2, and "E-like" strains 34-1, and GB-3 were purified by the method of Sacks and Alderton (1961) before recording spectra.

Saratoga and Beluga strains of Cl. botulinum Type E showed 90.5% and 80.7% sporulation respectively. The spectra of these spore cultures were of type ββ. Beluga (vegetative cells) was type β in the two day old agar cultures; however, Saratoga (vegetative cells) was type α. Strain VH#2 was type β; however, VH#2 spores were type δδ. Cl. butyricum was type α twice, and β once, but the spores were of type δδ (69% sporulation). Cl. botulinum "E-like" Strain 34-1 was type α, but its spores were type δδ. Cl. botulinum "E-like" Strain GB-3 was of type γ, but its spores were type δδ. (Compare Tables 25 and 28.) (See also Tables 1 and 3.)

An examination of Figures 30-35, along with the above information, indicates that the spore type may vary considerably from the vegetative cell; however, no distinctly new types of spectra are seen. The δδ pattern seems to be seen, except where it is overridden

by the ββ glycogen pattern. It would seem that the only appreciable differences between the spectra of spores and those of vegetative cells are those due to washing.

Table 28. Spectral types of the two day old clostridial spores grown in biphasic culture at 28°C

Organism	Spectral #	Spectral pattern	% Spores
Cl. botulinum Strains			
Saratoga	351	ββ	90.5%
Beluga	350	ββ	80.7%
VH#2	357	δδ	100.0%
34-1	354	δδ	100.0%
GB-3	356	δδ	100.0%
Cl. butyricum	352	δδ	69.0%

Figure 30. Spectra of spores and vegetative cells

Cl. botulinum type E strain Beluga

Spectral #	Cultural conditions	Age
#142	TPGY Agar (28°C) veg. cells	2 days
#143	TPGY Agar (28°C) veg. cells	2 days
#86	TPGY Agar (28°C) veg. cells	2 days
#350	TPG Biphasic Spore Culture (28°C)	2 days

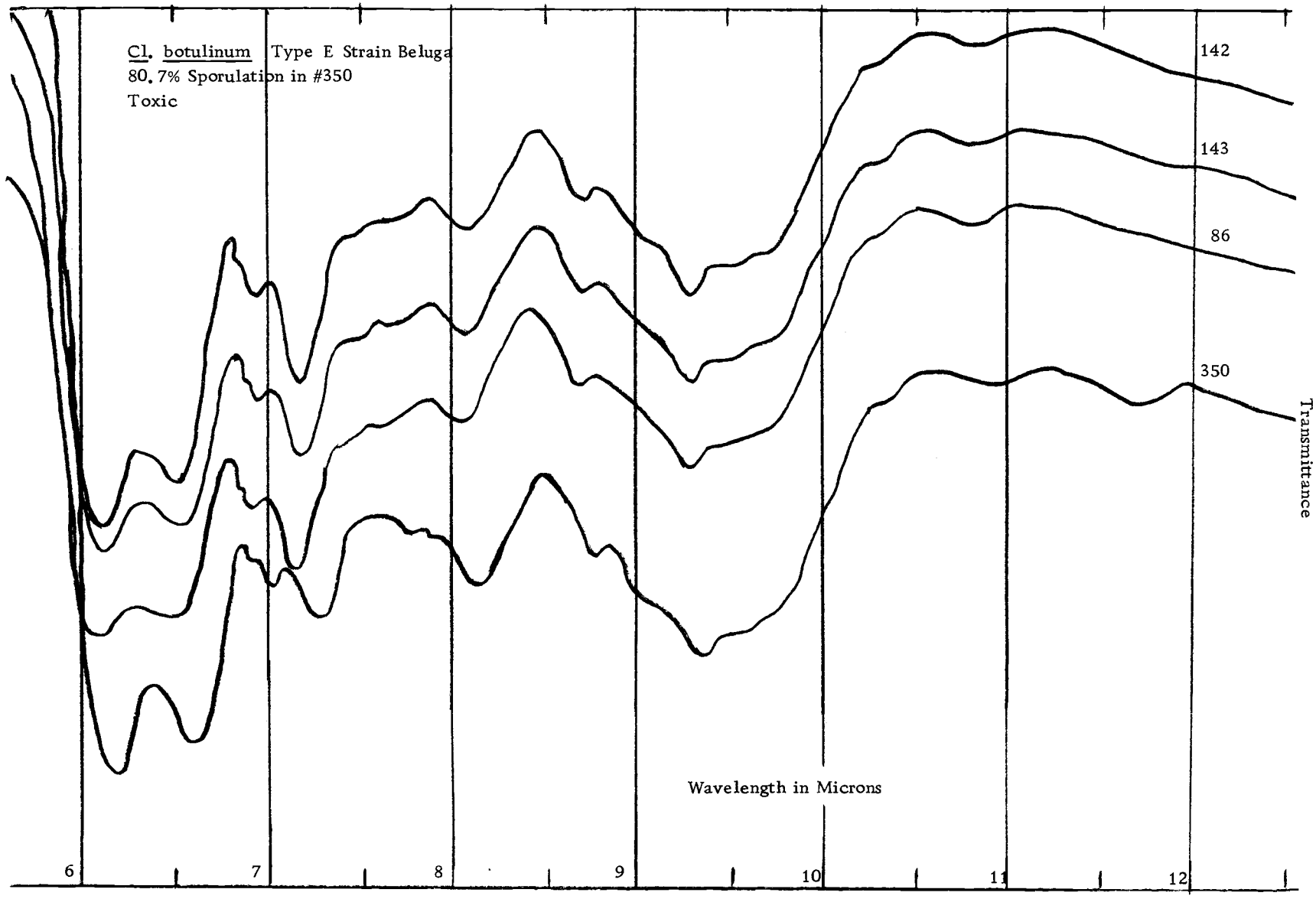

Cl. botulinum Type E Strain Beluga
80.7% Sporulation in #350
Toxic
142
143
86
350
Transmittance
Wavelength in Microns
6
7
8
9
10
11
12

Figure 31. Spectra of spores and vegetative cells

Cl. botulinum type E, strain VH#2

Spectral #	Cultural conditions	Age
#156	TPGY Agar (28°C) veg. cells	2 days
#157	TPGY Agar (28°C) veg. cells	2 days
#90	TPGY Agar (28°C) veg. cells	2 days
#357	TPG Biphasic Spore Culture	2 days

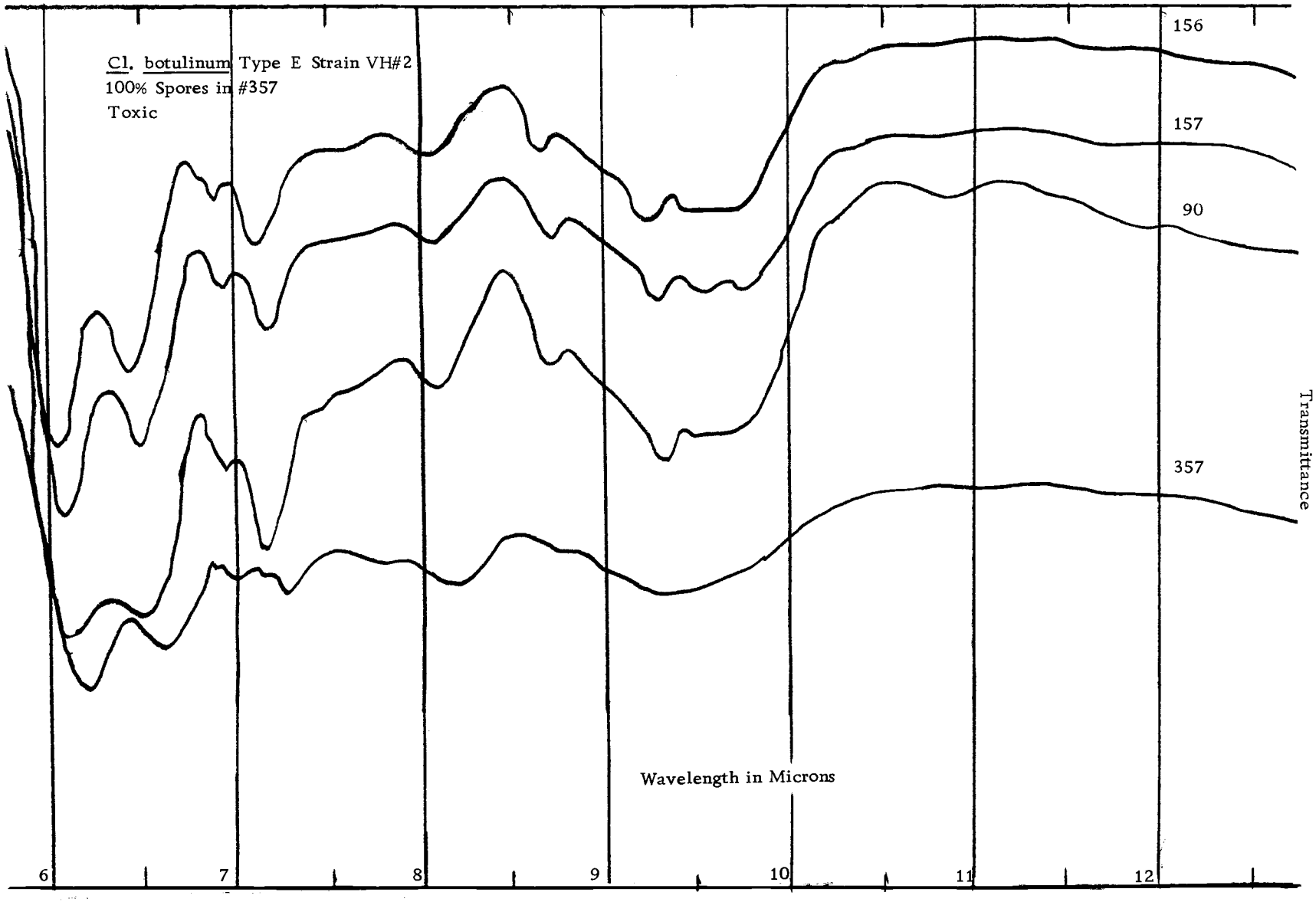

Cl. botulinum Type E Strain VH#2
100% Spores in #357
Toxic
156
157
90
357
Transmittance
Wavelength in Microns
6
7
8
9
10
11
12

Figure 32. Spectra of spores and vegetative cells

Cl. botulinum type E, strain Saratoga

Spectral #	Cultural conditions	Age
#144	TPGY Agar (28°C) veg. cells	2 days
#146	TPGY Agar (28°C) veg. cells	2 days
#82	TPGY Agar (28°C) veg. cells	2 days
#351	TPG Biphasic Spore Culture	2 days

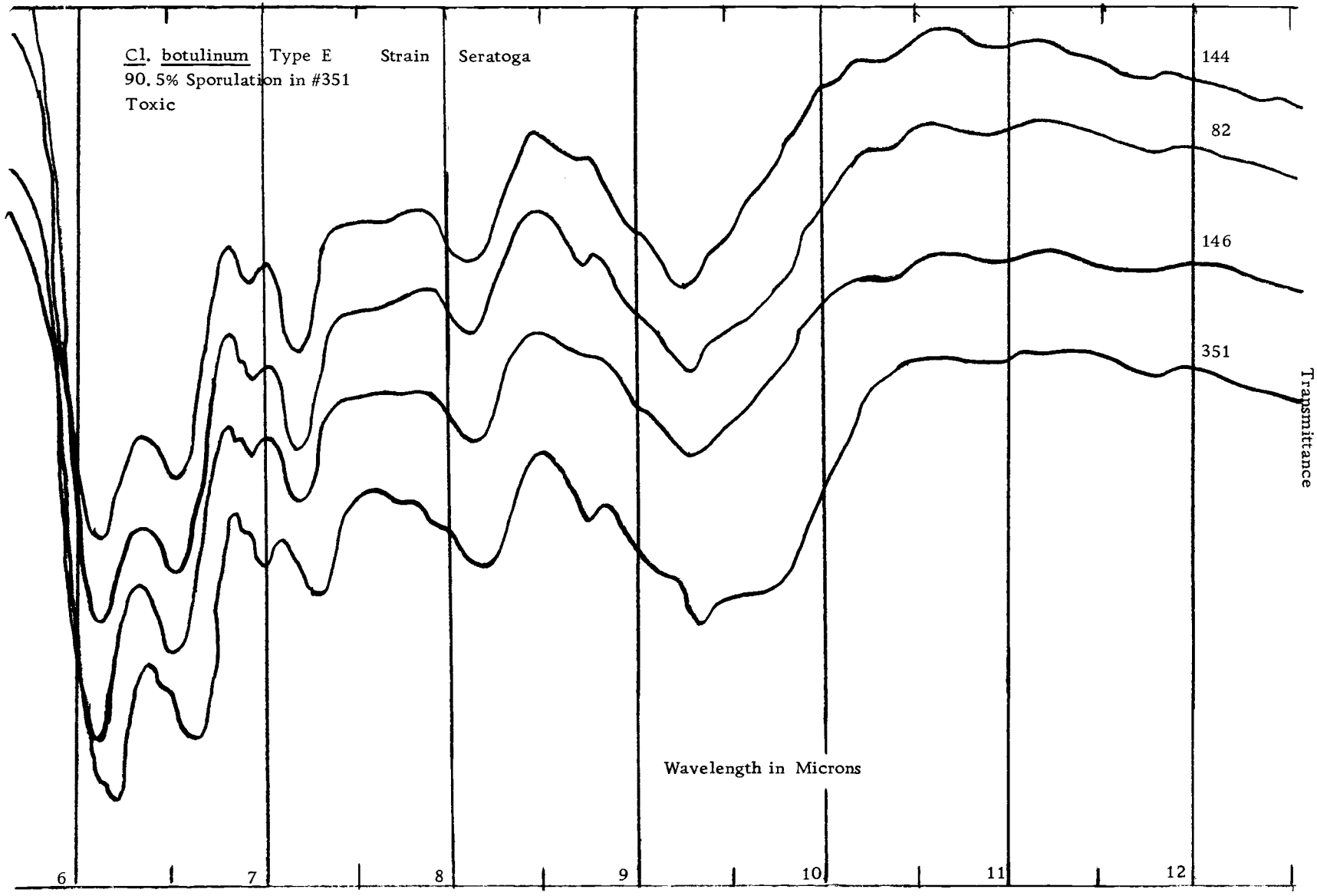

Cl. botulinum Type E Strain Seratoga
90.5% Sporulation in #351
Toxic
144
82
146
351
Transmittance
Wavelength in Microns
6
7
8
9
10
11
12

Figure 33. Spectra of spores and vegetative cells

Cl. butyricum

Spectral #	Cultural Conditions	Age
#94	TPGY Agar (28°C) veg. cells	2 days
#295	TPGY Agar (28°C) veg. cells	2 days
#93	TPGY Agar (28°C) veg. cells	2 days
#273	TPGY Agar (28°C) veg. cells	2 days
#352	TPG Biphasic Spore Culture	2 days

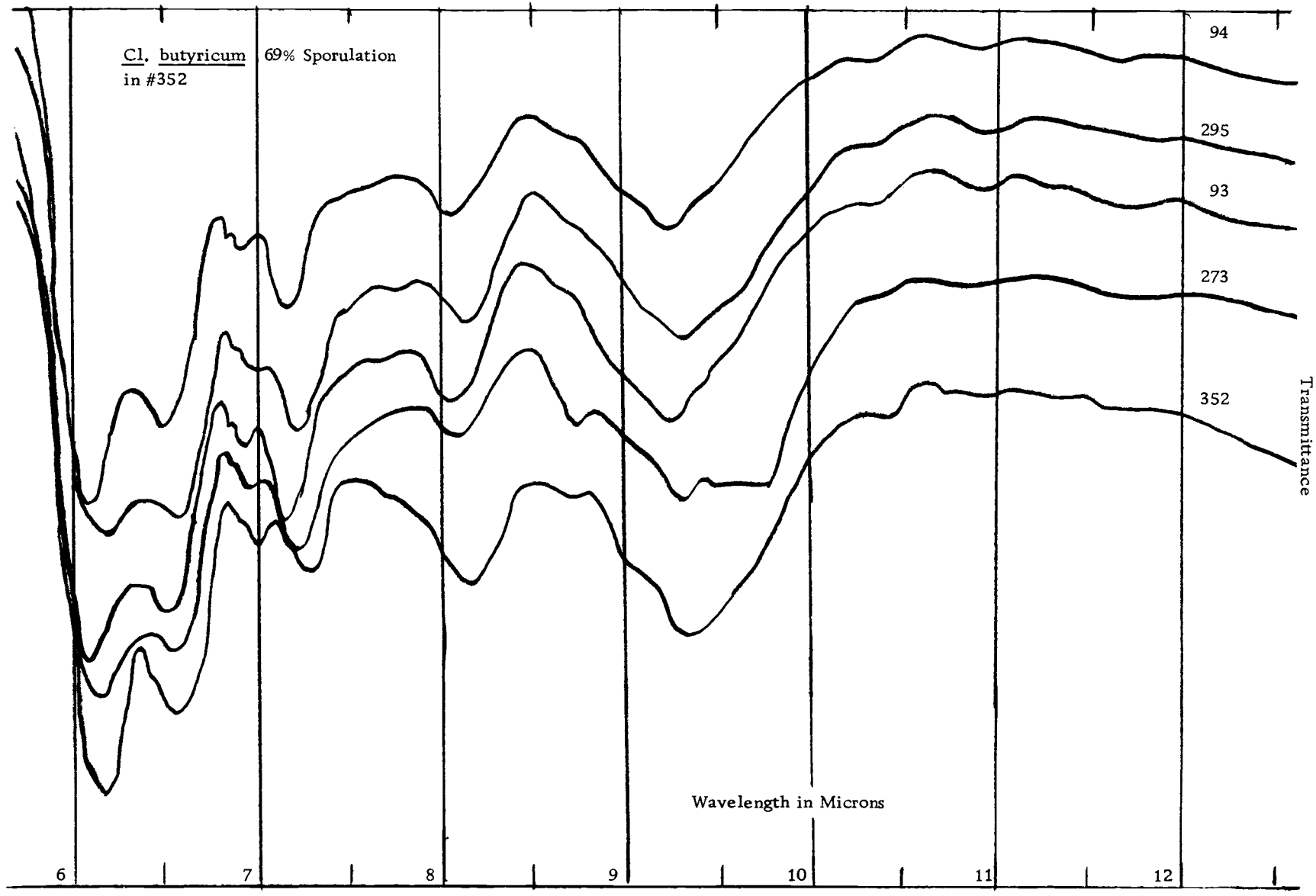

Cl. butyricum in #352
69% Sporulation
94
295
93
273
352
Transmittance
Wavelength in Microns
6
7
8
9
10
11
12

Figure 34. Spectra of spores and vegetative cells

Cl. botulinum "E-like," Strain 34-1

Spectral #	Cultural conditions	Age
#166	TPGY Agar (28°C) veg. cells	2 days
#167	TPGY Agar (28°C) veg. cells	2 days
#354	TPG Biphasic Spore Culture	2 days

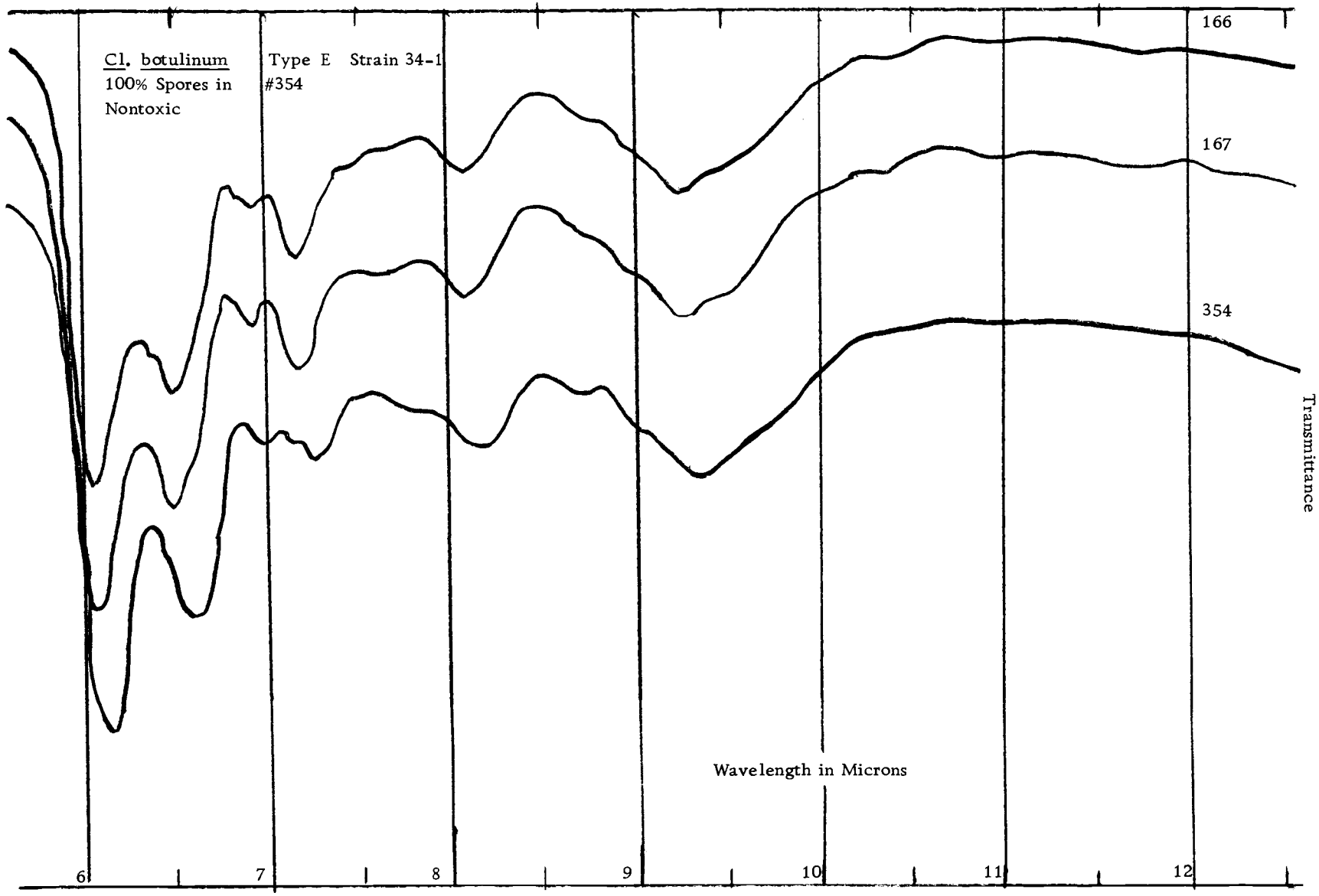
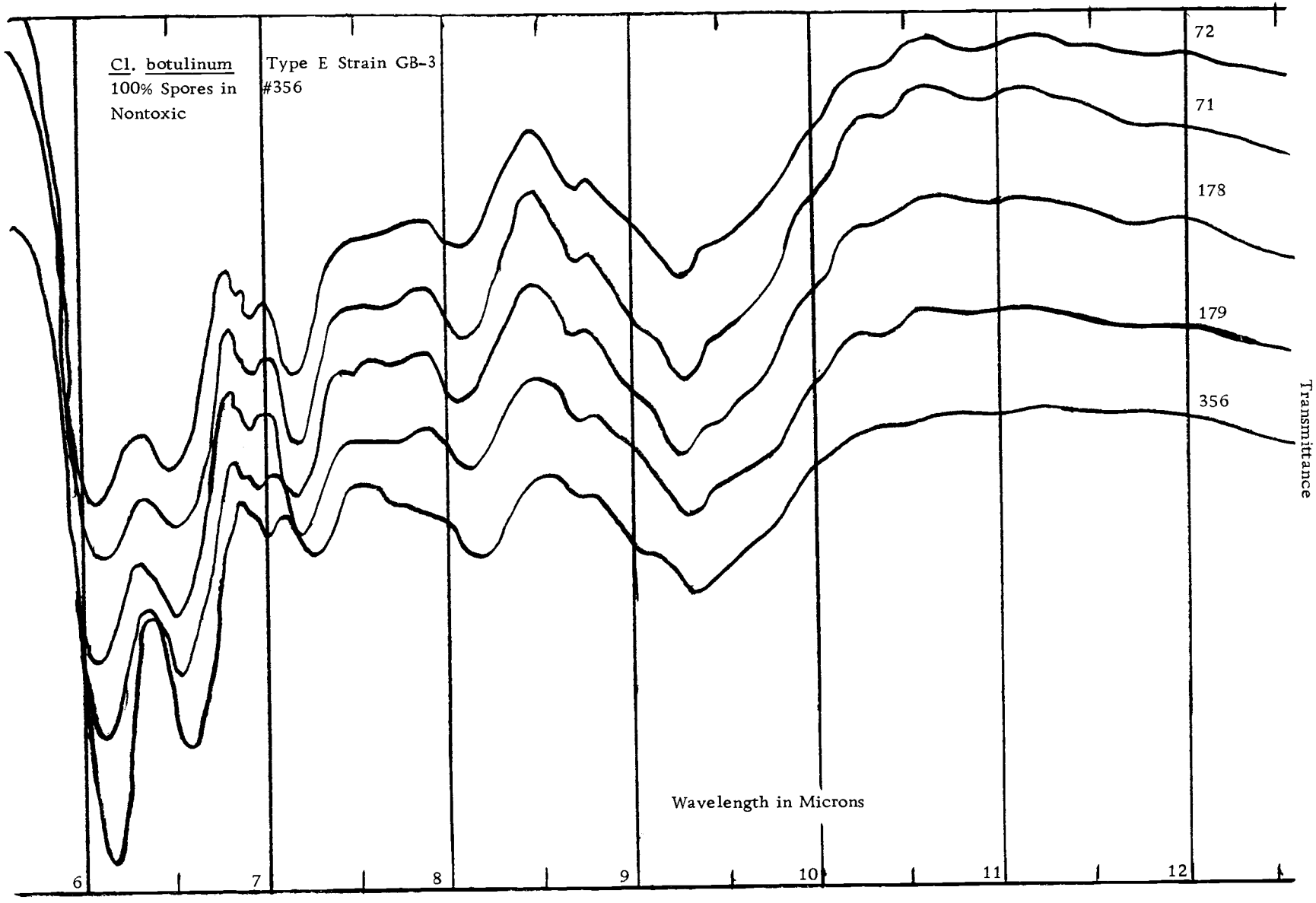

Cl. botulinum
100% Spores in
Nontoxic
Type E Strain 34-1
#354
166
167
354
Transmittance
Wavelength in Microns
6
7
8
9
10
11
12

Figure 35. Spectra of spores and vegetative cells

Cl. botulinum "E-like," Strain GB-3

Spectral #	Cultural conditions	Age
#71	TPGY Agar (28°C) veg cells	2 days
#72	TPGY Agar (28°C) veg cells	2 days
#178	TPGY Agar (28°C) veg cells	2 days
#179	TPGY Agar (28°C) veg cells	2 days
#356	TPG Biphasic Spore Culture	2 days


Cl. botulinum
100% Spores in
Nontoxic
Type E Strain GB-3
#356
72
71
178
179
356
Transmittance
Wavelength in Microns
6
7
8
9
10
11
12

DISCUSSION

It can be seen that a number of variables can enter into the composition of an infra-red absorption pattern for bacterial cultures. The spectra, even under identical conditions of culture media, incubation temperature, age, and oxygen tension may not be completely reproducible. Minor inflections can be seen from spectrum to spectrum. A cell in the pregrowth phase may give a different pattern than a cell in the log phase, and a cell in the log phase may give a different spectrum from one in the death phase.

This is amply brought out by observing the cultures giving the ββ glycogen type spectrum. Glycogen is a storage product, and the extent of its storage will depend on whether there actually is enough food so that it may be stored in this form. It can be seen from the series of spectra of two day old cells of Cl. botulinum, strain type E 15Ati, that a single strain may show great variability in its ability to store glycogen (Figure 4). Many members of the genus Bacillus store large amounts of polymerized β hydroxy butyric acid (Blackwood, 1957, and Haynes, 1958). This shows up very vividly in the infra-red spectrum of the organism. (See Spectral Pattern H, Figure 15.) This pattern also is not constant, and a culture may only exhibit it at certain phases of its growth.

In general, the variations which are seen between the spectra

of the same organism, grown to the same age under the same conditions are much more subtle than this. However, the variations in the spectral patterns used in classification and physiological measurement are equally subtle. Extreme caution must be used, therefore, when making inferences about an organism from its infra-red absorption patterns.

Additional variability may be seen from variation in smear thickness. The classical method of applying the bacterial smears to the windows is to scrape a small amount of growth from a petri plate with a rubber policeman, and dissolve it in a drop or two of water water on a AgCl disc, smear evenly over the surface of the disc and allow to dry over night. The smear thickness may, therefore, vary greatly, and is dependent only upon the manual dexterity of the worker. In the light of the crudity of the application procedure, it is indeed amazing that any reproducibility whatsoever is achieved.

Upon looking at the variation between a smear which is too thick or too thin, as compared with a smear which has its spectrum approximately centered on the graph paper, it is evident that both the thick and the thin smear give washed out flattened spectral patterns which make comparison very difficult. Indeed, in a thick smear, the entire double peaks at 6.1 μ and 6.5 μ may appear as one solid broad absorption band. In a thin smear, the shape of the pattern between 8.5 μ and 10.5 μ may be so washed out as to make it

completely undecipherable. This spectral range from 8.5 μ to 10.5 μ is the critical range for the determination of spectral patterns.

The silver chloride windows are very soft. Even lens tissue will scratch them. It is not long, even under the most careful conditions, before the worker finds his AgCl windows covered with tiny scratches. These scratches will cause light scattering and differential absorption. Fortunately, the scratch must be large, compared with the magnitude of the wavelength of the light itself to really be effective in light scattering (Jones, 1952). In addition to this, it is found that the silver chloride windows will darken with age. This darkening process appears to proceed at different rates with different windows. It may be readily seen by taking the IR spectrum of the empty window, using air as a reference. The darkening process is most evident in the shorter ranges of the spectrum (from about 2 to 8 μ). It appears to be a smooth and gradual darkening; however, and does not appear to have any peaks or troughs to distort the spectrum of the bacterial smear. This darkening process may be partially corrected for by using a clean dry silver chloride window in the reference beam of the spectrophotometer. This must be done with reservation; however, for no two windows are exactly alike, have the same scratches, or have darkened to the same extent.

The dried bacterial film on the windows is a heterogenously dispersed and particulate system, which has been shown not to follow

Beer's Law (Jones, 1952). The saving factor here is that the size of the bacteria themselves is small when compared with the wavelength of light with which they are examined.

Care must be used only to compare organisms of the same culture age, for as shown in the experiments with Cl. botulinum "E-like" strain 170C (see Figure 18), changes do indeed occur, with age, in the IR spectrum of a culture. These changes are of such a magnitude that they may remove an organism from one spectral pattern and place it in another.

The washing process greatly alters the infra-red absorption spectrum of the cells, as shown in the proceeding experiments. It is shown that the area of the spectrum from 6.1 μ to 8.1 μ is altered to the greatest extent by the washing process. This area is attributed to the types of bonds found in amino acids, polypeptides, and proteins (C=O stretching, CONH, N-H deformation, and peptide link vibrations). The area from 8.5 μ to 10.5 μ is also altered to a great enough extent to change the organism from one spectral pattern to another.

The bonds absorbing in this area are C-O-C vibrational modes, C-O stretching vibrations, and OH deformations. These are the types of bonds found in polysaccharides and nucleic acids.

It would seem that great quantities of amino acids, polypeptides, proteins, and lesser quantities of polysaccharides are removed from

the cell during the washing process. The question may be logically raised as to whether this material on the outside of the cell consists of nutrients from the medium which are adhering to the cell, or whether it represents metabolic products or byproducts of the cell. It may be possible that material is leached out of the cell itself. Perhaps it is a combination of all of these. This in turn raises the question as to whether this material should be allowed to remain and be counted as part of the spectrum of the cell or whether it should be washed away before the spectrum is recorded. If a difference between the cells is searched for on the basis of a difference in their metabolic excretions, such as a difference between the toxigenic and nontoxigenic strains of Cl. botulinum, then it would indeed be desirable to leave the cells unwashed. This may, however, cause additional problems, such as unreliability of the calculations of glycogen concentration, as given by Levine (1953). These calculations depend on the ratio of the OD at 9.75 μ to the OD at 6.5 μ. The 6.5 μ OD is well within the 6.1 μ to 8.1 μ range which is greatly altered by washing, however.

Despite all of the possible sources of error in the interpretation of the infra-red spectra of micro organisms, the basic composition of the cell is clearly evident from its IR absorption pattern. Certain large peaks are noted, and these are present in all microbial IR absorption patterns. The type of bonds giving rise to these

absorption peaks are found within the chemical compounds which are most common within the cell. These are proteins, nucleic acids, polysaccharides, lipids, and water. The other substances which are present in the cell are present in such small quantity that they do not show up on the spectrum. Only when a certain substance is present in such quantity that it is able to override the basic spectral pattern of the microbial cell, will its presence be detected.

The type α spectral pattern is indicative of a young culture. As a culture ages, its pattern changes from spectral type α to spectral type γ or δ. The spectral pattern β seems to be especially characteristic of the VH#1, VH#2, and Beluga strains, which remained spectral pattern β throughout; all of the other toxic type E strains changed to the spectral pattern γ with age. The nontoxic "E-like" strains, except GB-3, 15Ati, and 066BNT, exhibited pattern γ at the five day age. Strains GB-3 and 15Ati were pattern β, while 066BNT was pattern λ twice, and pattern γ once. (See Table 7.) The γ pattern has a slight peak at 8.7 μ. If this peak is considered to be indicative of a small amount of glycogen formation, it is noted that 100% of the toxic type E strains of Cl. botulinum, 100% of the nontoxic type E strains, the one type F, and 43% of the miscellaneous clostridia (see Table 9) exhibit some sort of glycogen pattern.

This seems to be consistent with the findings of Stiebrs (1967), who reports the presence of starch-like granules in the type E

Clostridium botulinum when grown on TSA egg yolk medium. She finds that the colonies turn blue, and the individual organisms exhibit dark granules when the plates are flooded with iodine. She concludes that this is a method of distinguishing type E organisms.

It then remains to be determined whether the substance accumulated within the cells of the type E strains is starch or glycogen. A wet chemical test should be initiated to make this determination. It would be interesting to determine the infra-red spectra of cells grown on TSA egg yolk medium as opposed to TPGY media.

The spectral patterns of the miscellaneous clostridia seem to change from two days to five days from type α pattern to either patterns γ or δ. The γ and δ patterns seem to be indicative of types A, B, and F at both two and five days. The significance of the spectral pattern δ is not immediately apparent. Cl. botulinum type F consistently exhibited spectral pattern γ, while 113B, 115B, 5A, and 33A consistently exhibited the δ pattern at two days and five days.

The investigation of spores did not seem to turn up anything of significance.

A comparison of the miscellaneous bacteria would indeed indicate that they can be distinguished from the clostridia on the basis of their spectral patterns. The spectral patterns G, H, I, and J seem to be uniquely associated with these organisms. This is especially true of pattern H. Since such a small sample of miscellaneous

bacteria was tested, extreme caution should be made in drawing inferences from this.

SUMMARY

There are six immunological types of Cl. botulinum (Types A, B, C, D, E, and F. The differentiation between these types is based upon the antigenic structure of the toxin. Many strains of these various types have been isolated. In addition, a number of nontoxigenic type "E-like" strains have come to light. It would be of great value to have a rapid method of distinghishing between these strains, or of identifying the type of a toxigenic culture. This thesis discusses an investigation into this problem. A cross section of the IR spectra of other important clostridia is also considered.

Smears of bacterial cells were prepared by scraping a small amount of growth from the agar surface of a petri plate, and dissolving it in a drop of water on the surface of a silver chloride disc. In the case of broth cultures, growth to be placed on the discs was first obtained by centrifugation. A second empty disc was then placed over the first with a small bit of lens tissue under one edge to prevent the formation of interference fringes. The two discs were then taped together and placed in the IR spectrophotometer, together with a pair of reference discs, for the recording of the IR spectra of the whole bacterial cells.

1. Before any work could be initiated on the spectra of the various organisms, some preliminary information as to the reproducibility

of the spectra was needed. Upon repeated examination of the same strains, it was found that the most reproducible section of the IR spectrum, which would also give unique and somewhat characteristic variation was to be found in the wavelength region from 7.0 μ to 10.0 μ.

2. Some preliminary information on the effect of bacterial smear thickness was also needed. By recording and examining the spectra made from smears of various thicknesses, it was found that the characteristic variations of an organism were magnified to the greatest extent when the smear was of such a thickness that the 7.0 μ to 10.0 μ region of the spectrum had its entire transmittance lying in the range from 20% to 70%. Since no known rapid and quantitative technique of bacterial smear preparation was known, it became somewhat of an art to obtain smears of the desired thickness.

3. It was seen that the IR spectra of the great bulk of the clostridia fell into four basic patterns (α, β, γ, and δ). The other bacteria examined seemed to give thir own patterns, different from the above four.

4. The age of the culture seemed to have a rather profound effect on the IR spectrum of a culture. The variability of the IR spectral pattern of a culture decreased with age, between two and five days. It is interesting to note that the type α spectral pattern completely disappeared at the five day age, although it was present as the basic

spectral pattern of 51.2% of the two day old cultures examined. The number of strains exhibiting pattern γ is seen to greatly increase at the five day age. The significance of this seems to be that a small amount of glycogen accumulates in the cells of a culture with age. This theory is dependent upon the assumption that the gradual development of the 8.7 μ peak, with age, is indicative of glycogen accumulation. The development of this 8.7 μ peak is especially noted in the type E strains of Cl. botulinum.

5. It is concluded that the technique has little value as an aid in the identification of clostridial species. Most of the pathogenic clostridia could not be distinguished from the non pathogens by this means. Neither could the toxigenic strains of Cl. botulinum type E be distinguished from the non toxigenic 'E-like" strains. The technique may have some value in monitoring physiological changes within a given culture or group of cultures, however.

6. Washing the culture before preparing smears had a rather large effect on the spectrum. The entire region of the spectrum from 6.2 μ to 8.2 μ was raised or considerably decreased in intensity from the rest of the spectral curve. (See Figures 27-29.) This is the section of the spectrum associated with proteins. Changes also occurred in the region of the IR spectrum from 8.5 μ to 10.5 μ after washing. This region is associated with nucleic acids and polysaccharides.

7. Examination of purified and unpurified spore preparations

indicates that the IR spectra of the spores may vary from the vegetative cell, but no distinctly new types of spectra are seen.

It appears that the main differences seen between the spectra of spores and those of vegetative cells are due to washing.

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