THE TEMPERATURE RELATIONS OF *CLOSTRIDIUM BOTULINUM*, TYPES *A* AND *B*

By D. F. Ohye* and W. J. Scott*

[*Manuscript received November* 25, 1952]

Summary

Ten strains of *Cl. botulinum,* type *A,* and 10 of type *B* have been studied at 12 temperatures between 10 and 50°C., and rates of growth measured nephelometrically on sealed cultures. Growth proceeded from spore inocula at temperatures from 15 through to 42.5°C., but not at 12.5 or 45°C. When young, actively growing cultures were transferred to temperatures outside the range permitting spore germination, rates of growth were measured at 12.5, 45, and 47.5°C. After transfer to 10 or 50°C. no sustained growth was observed.

The temperature coefficient of the mean rates of growth was very large at temperatures less than 15°C., gradually decreased to zero at about 40°C., and became negative at higher temperatures. With spore inocula the deduced lag periods were least at 37°C., slightly greater at 42.5, and very much greater at temperatures less than 20°C. The total yield of cells was greatest at about 37°C. and fell considerably as the upper and lower temperature limits were approached. The 20 cultures studied formed a reasonably homogeneous group in their reactions to temperature.

I. Introduction

The extensive, but largely qualitative, observations by early workers led to considerable differences of opinion regarding the most favourable temperatures for growth and toxin production by *Cl. botulinum.* This early work has been reviewed by Meyer (1928) who has also included some of his own results. Meyer has stated that the optimum temperature lies between 25 and 38°C. and is usually about 35°C. The range over which growth may take place is given as 15-55°C.

Since 1928 few additional papers dealing with the effects of temperature on *Cl. botulinum* have been published. Meyer and Gunnison (1929) in a paper on European strains state that growth was equally good at 20 and 37°C., although the lag was greater at the lower temperature. Tanner and Oglesby (1936) studied 21 strains (seven type *A* and 10 type *B*) at 5, 10, 15, and 20°C. Using vegetative inocula they found that all strains grew at 15 and 20°C. At 10°C. there was some growth of five strains although the results were marred by a temporary rise of the incubator temperature to 20°C. No growth occurred at 5°C. When cultures were inoculated with spores, 18 strains grew at 20°C. and only three at 15°C. No development took place at 5 or 10°C. during 10 weeks incubation.

* Division of Food Preservation and Transport, C.S.I.R.O., Homebush, N.S.W.

Tanner and Oglesby concluded that different strains of *Cl. botulinum* "vary materially in their temperature relations" but it is doubtful whether their results actually support such a conclusion. Haines and Elliott (1944) considered the possibility of toxin production at relatively high temperatures. Using six strains (four type *A* and two type *B*) they observed "no appreciable growth" in either of two media over a period of 14 days at 45°C., although six strains of *Cl. sporogenes* all grew within 24 hr. at this temperature. The type of inoculum was, however, not specified. All six strains of *Cl. botulinum* grew well and produced toxin at 37°C.

Although Haines and Elliott studied the time required for detectable amounts of toxin to be liberated at 37°C., they presented no data on the rate of growth of the organism. Mason (1935) quotes a mean generation time of 35 min. as the maximum rate of growth observed for *Cl. botulinum*. This figure was taken from some data on viable counts made by Wagner, Meyer, and Dozier (1925), who had incubated a veal infusion, peptone, glucose broth at 37°C. The data presented by the latter authors are, however, not very suitable for evaluating rates of growth as samples were taken at intervals of 6 hr. and very few of the estimates lie within the logarithmic phase of growth.

It appears that there is a general lack of data on the rates of growth of most species of *Clostridium* and very few figures are available for *Cl. botulinum*. The present paper reports the rates of growth observed when 20 strains of *Cl. botulinum* (10 type *A* and 10 type *B*) were studied at 12 temperatures between 10 and 50°C.

II. Methods

Cultures were grown in Pyrex test tubes (150 by 15 mm.) sealed under vacuum. The cleaned tubes were first drawn out to a neck, sterilized, and 10 ml. of sterile medium added aseptically. The tubes were sealed immediately after inoculation. Sealing was facilitated by carefully drawing down the neck of the tube to an internal diameter of 1-2 mm. before applying the vacuum. The contents were then boiled under vacuum for a few seconds and the seal completed. The risk of contamination by non-sporing organisms was eliminated by heating the sealed tubes for 10 min. at 80°C. immediately after inoculation.

The light scattered by each tube was then measured in a photoelectric nephelometer and the tube was permanently marked so that it could be replaced in the instrument in the same position. The nephelometer contained a vacuum-type photo-tube collecting light scattered at about 45° from the incident beam, the output of the photo-cell being amplified and measured on a 0-250 D.C. micro-ammeter. The range of the instrument was increased about 80-fold by a selector switch giving five ranges of sensitivity on the micro-ammeter. Readings on blank tubes usually varied between 20 and 50μA. on the most sensitive scale, whereas readings of fully grown cultures were equivalent to some 5000-8000 μA. For readings up to about 5000 μA. the nephelometer reading was directly proportional to the bacterial concentration. Rates of growth (R) were taken as the slopes of the straight lines obtained when logarithms of nephelometer readings (minus the blank values) were plotted

against time. Values of R are expressed in generations per hour and the lag is evaluated by assuming that R is constant (Monod 1949). Calibration curves with eight strains grown at 20, 30, or 37°C. showed that in the linear range each μA. was equivalent to approximately 4.2×10^{-2} μg. of dry cells or $1.75 \pm 0.07 \times 10^5$ cells.

Tubes for incubation were completely immersed in water-baths thermostatically controlled (± 0.05°C.) at the appropriate temperature. For a few of the earlier experiments at intermediate temperatures air thermostats (± 0.5°C.) were used. Baths maintained at temperatures less than 20°C. were operated in cool rooms. This avoided any condensation on tubes which would affect nephelometer readings, and also prevented any possibility of the cultures being temporarily above the temperature of the thermostat.

The medium (N.Y.G.) consisted of 10 g. of neopeptone (Difco), 5 g. of yeast extract (Difco), and 5 g. of glucose dissolved in 1 l. of glass-distilled water and adjusted to pH 7.0. Some experiments were also made with peptic digest (P.D.) medium and cooked meat medium (C.M.M.) (Dubovsky and Meyer 1922). Cultures were inoculated with 10,000 toxin-free viable spores contained in 0.1 ml. phosphate buffer, i.e. 1000 spores per ml. of medium.

Growth outside the temperature range for spore germination was studied in cultures transferred, in the early logarithmic phase, from more favourable temperatures. As the sealed culture was transferred the effects of the temperature change were not complicated by a disturbance of the anaerobic conditions.

Twenty cultures have been studied at all temperatures. Ten of these (3, 33, 38, 62, 109, 134, 172, *L*4, *Q*6, and *Q*7) were type *A* and ten (34, 112, 169, 213, 968, 1787, 3149, *A*12, *L*12, and *T.B.*) were type *B*. The first seven of the type *A* strains and the first four of the type *B* strains were obtained from Prof. K. F. Meyer.

III. Results

(*a*) *Rates of Growth*

Growth occurred from spore inocula at all temperatures between 15 and 42.5°C. although the spores of two (type *B*) strains failed to germinate at 15°C. and six (three type *A* and three type *B*) failed at 42.5°C. Growth of all 20 strains proceeded from spore inocula at all temperatures between 17.5 and 37°C. On the other hand none of the strains grew from spore inocula at 12.5 or 45°C. At 12.5°C. eight (three type *A* and five type *B*) strains grew when spores were germinated and growth was commenced at 20°C., whereas at 45°C. 15 (nine type *A* and six type *B*) strains grew when growth was initiated at lower temperatures. With some of the 15 strains which grew at 45°C. the logarithmic phase was not well defined and steady rates of growth were recorded for only 11 strains (seven type *A* and four type *B*). Five of the strains (three type *A* and two type *B*) which grew at 45°C. also grew at 12.5°C. The mean rates of growth and their standard errors at various temperatures are given separately for each toxigenic type in Table 1. It may be seen that the mean rates are almost identical for the two types at 20 and 25°C. At 30 and 37°C. the mean rates for type *B* strains are slightly greater than for type *A*. At all

other temperatures the mean rates are slightly greater for type *A* strains. None of the differences was found to be significant ($P > 0.05$) and, moreover, the variances were similar for each group. The data for both toxigenic types have, therefore, been grouped together.

TABLE 1

MEAN RATES OF GROWTH FOR TYPES *A* AND *B* STRAINS AT VARIOUS TEMPERATURES IN N.Y.G. MEDIUM

Temperature (°C.)	Type *A* Strains			Type *B* Strains		
	No. of Strains	Mean Rate (Divisions/ hr.)	S.E. of Mean	No. of Strains	Mean Rate (Divisions/ hr.)	S.E. of Mean
12·5	3	0·0119	0·0024	5	0·0107	0·0008
15·0	10	0·0359	0·0058	8	0·0346	0·0084
17·5	10	0·131	0·0080	10	0·128	0·0112
20·0	10	0·260	0·0151	10	0·259	0·0208
25·0	10	0·535	0·0166	10	0·533	0·0156
30·0	10	0·917	0·0263	10	0·990	0·0356
37·0	10	1·485	0·0662	10	1·576	0·0492
42·5	7	1·305	0·147	7	1·076	0·137
45·0	7	0·533	0·068	4	0·477	0·074

The average values of R for all strains in N.Y.G. medium are shown in Figure 1 for temperatures between 12.5 and 45°C. At the two extremes the rates are, of course, based on experiments with vegetative inocula. At intermediate temperatures the rates are from experiments where spore inocula were used. When all strains grew the value of R is the arithmetic mean for 20 strains, but at temperatures where some strains failed to grow the mean is based on the smaller number which grew. The standard error of the mean, expressed as a percentage of the appropriate mean rate, is under 3 per cent. at 30 and 37°C., and rises to between 10 and 12 per cent. at temperatures approaching the upper and lower limits for growth. The variation between the minimum and maximum rates at any one temperature is considerable and amounts to some 30-40 per cent. of the mean at temperatures between 25 and 37°C. As the temperature limits for growth are approached the variation increases and the range exceeds the mean at 15 and 45°C. The mean value of R is shown to have a maximum of approximately 1.62 at a temperature close to 40°C. Although no experiments were performed at 40°C. the value of R at this temperature is supported by plots of log R against the temperature or the reciprocal of the absolute temperature.

Also shown in Figure 1 is the temperature coefficient $1/R.dR/dt$ plotted against the temperature (t). This curve is derived from the values of R taken at intervals of 1°C. from the other curve in the figure. It will be noted that

the temperature coefficient becomes very large as the minimum temperature is approached. For instance, at 15°C. the rate of growth will be increased by about 50 per cent. per degree rise in temperature, and at 12.5°C. the figure is about 73 per cent. The corresponding values of Q_{10} are approximately 58 and 240. The effects of small changes in temperature are also large as the maximum temperature is approached, the coefficient in this range being negative.

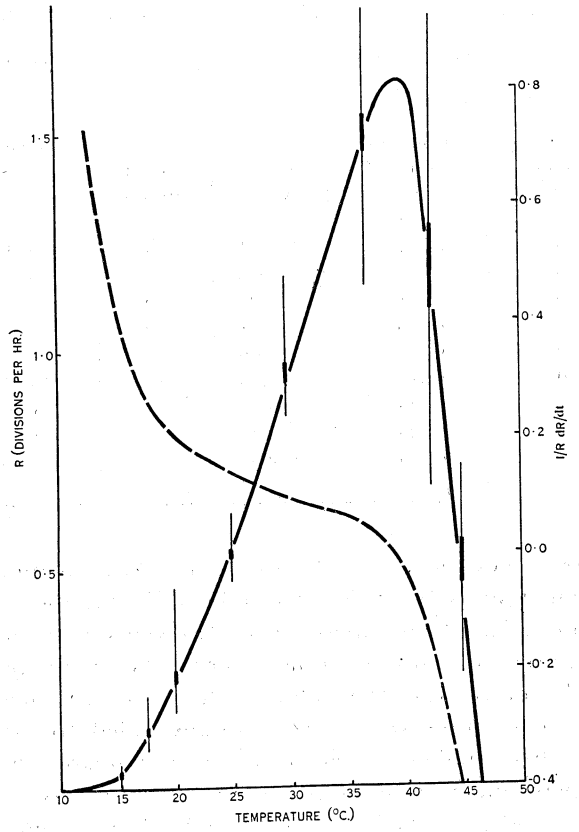

Fig. 1.—Relation between the mean rate of growth (R), the temperature coefficient of R, and the temperature (t) in N.Y.G. medium. (The heavy vertical lines show the mean value of $R \pm$ its standard error; the light vertical lines show the range of values observed at each temperature.) ———— Rate of growth. — — — — Temperature coefficient.

(*b*) *Lag*

The duration of the lag period, which includes the time required for germination of the spores, was found to be markedly dependent on temperature. The average results for all experiments are shown in Figure 2 in which the logarithm of the lag is plotted against temperature. The average lag was

found to be about 5 hr. at 37°C., over a day at 20°C., and almost a week at 15°C. The value at 42.5°C. was significantly greater than at 37°C. At all temperatures there was close agreement between duplicate tubes and fair agreement between strains, but a larger variation between experiments carried out on different occasions. It may be concluded, therefore, that uncontrolled variations between different batches of medium had greater effect on the lag than variations in the condition of the inoculum, or in the degree of anaerobiosis effected during sealing of the tubes.

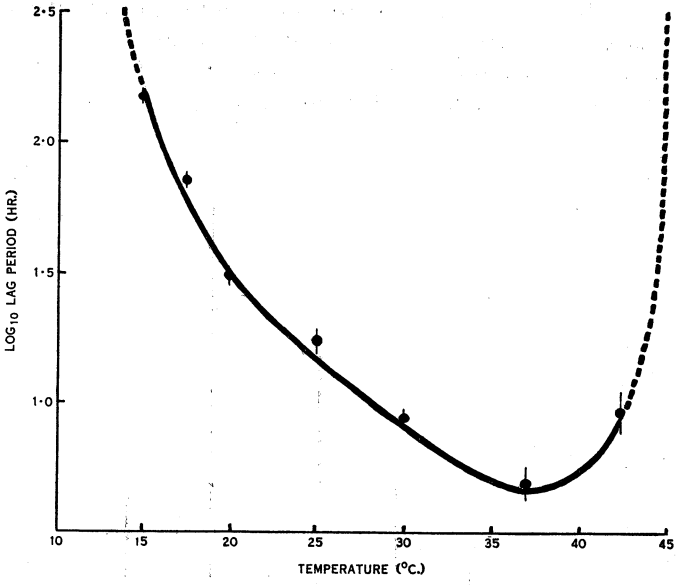

Fig. 2.—Relation between the lag period and temperature (N.Y.G. medium). The vertical lines indicate the mean ± its standard error.

(*c*) *Yield of Cells*

Data on the maximum size of crop were obtained from nephelometer readings taken in the early stationary phase. Readings during the late stationary phase were often as much as 70 per cent. less than the maximum readings. This phenomenon, which is presumably due to lysis, is much more pronounced, and has a more rapid onset, at the higher temperatures. Cells from cultures showing this fall in turbidity have frequently undergone partial disintegration, they stain only faintly, and show no evidence of incipient sporulation. The average results for all experiments are given in Figure 3. Yields of less than about 200 μg. dry weight per ml. are within the linear range of the measuring instrument, and will be somewhat more reliable than for the denser cultures where the dry weight-nephelometer relationship is no longer linear. The data are nevertheless adequate to demonstrate a marked effect of temperature on the total yield of cells.

(*d*) *Lower Temperature Limits*

In the present experiments almost all strains developed from a spore inoculum at 15°C. At this temperature 10 (five type *A* and five type *B*) of the cultures which grew were tested for the presence of toxin after incubation for 40 days. Titres of the order of 10^3-10^4 mouse-doses per ml. were observed with all the type *A* cultures. All but one of the type *B* cultures proved toxic to mice, the titre being of the order of 10-100 mouse-doses per ml. At 10 and 12.5°C. evidence was obtained that the spores did not germinate. After incubation at 12.5°C. for 3 months and after 6 months at 10°C. the sealed tubes were immersed in a water-bath at 80°C. for 10 min. Subsequently the tubes were transferred to 30°C. Growth occurred in all tubes after a normal lag. The original inoculum had, therefore, remained resistant to 80°C. and the medium was still suitable for the growth of *Cl. botulinum*.

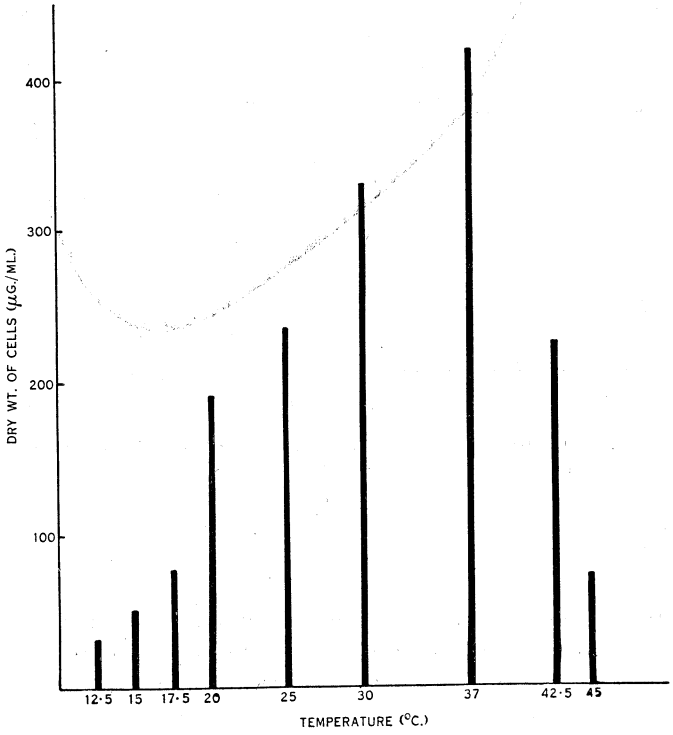

Fig. 3.—Relation between the yield of cells and temperature (N.Y.G. medium).

When growth was commenced at 20°C. and the tubes subsequently transferred, during the early logarithmic phase, to 12.5°C. growth of some strains continued at the lower temperature. As the tubes were completely immersed in the water-bath they would be cooled to within 0.1°C. of the lower temperature within 5 min. of the transfer. It was, nevertheless, often observed that during an initial period of some 2-8 hr. the rate of growth was much

greater than that maintained thereafter. During this initial period the rate of growth was sometimes almost as great as at 20°C., but the extent of growth was never greater than one generation and more usually only one-quarter to one-half of this amount. Subsequently, a much slower steady rate of growth was maintained over several days and it is this lower rate shown in Figure 1. The higher initial rate is presumably at the expense of metabolites or cellular components already accumulated during the early growth at 20°C. An indication of some of the different responses of cultures transferred to 12.5°C. is given in Figure 4. There is evidently need for caution in deciding which rate is appropriate to the steady state at 12.5°C., but it is suggested that the lower rates are the best values available at present.

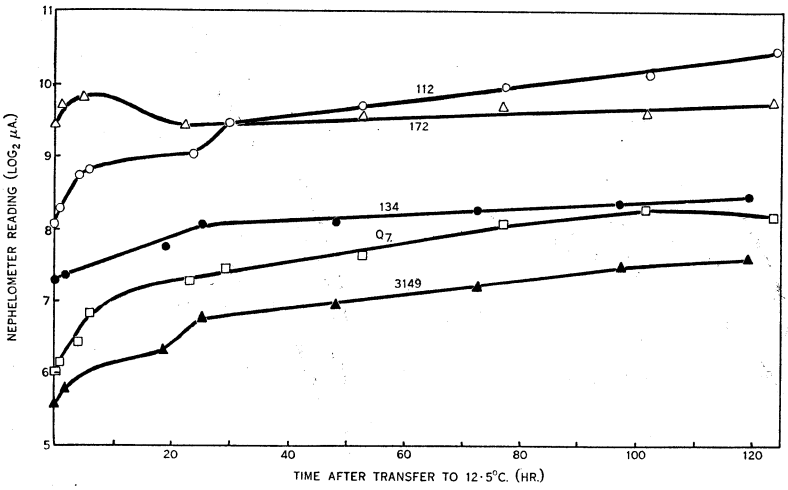

Fig. 4.—Growth of various strains at 12.5°C. after transfer from 20°C. (N.Y.G. medium).

When cultures were transferred from 20 to 10°C. there was little or no increase in the first few hours and subsequently very little change in turbidity during a period of 10 days. There was, however, a general tendency for a very slow increase during the first 5 or 6 days at 10°C. followed by constant or slowly declining readings of turbidity. Growth was too slow for rates to be measured reliably and the amount was limited to approximately 1.5 generations during the period of observation. It is believed that this small increase reflects the utilization of metabolites previously synthesized at 20°C. rather than a real capacity to proliferate at 10°C.

(*e*) *Upper Temperature Limits*

There was no growth from spore inocula in N.Y.G. medium held at 45°C. for 50 days, nor in P.D. medium held for 40 days. With both media the inoculum survived 10 min. at 80°C. after incubation, and growth occurred when the tubes were transferred to 37°C. The evidence is, therefore, that the spores had failed to germinate and that the media remained suitable for growth. Many

of these cultures were transferred back to 45°C. after growth had commenced at 37°C. Growth then ensued at 45°C. even though the spores had previously failed to germinate in these same tubes at the same temperature. When cultures in which growth had commenced at 30 or 37°C. were transferred to 45°C. the results were rather variable. In many cultures a high initial rate approximately equal to the rate at 37°C. was maintained for 1-2 hr. This was followed by a gradual or sometimes rather abrupt change to a lower constant rate. In other cultures the initial high rate was not detected or was evident to only a slight extent. Some growth curves illustrating the different types of response are shown in Figure 5. Both the media used showed similar types of response, but the rates of growth were somewhat higher on P.D. medium.

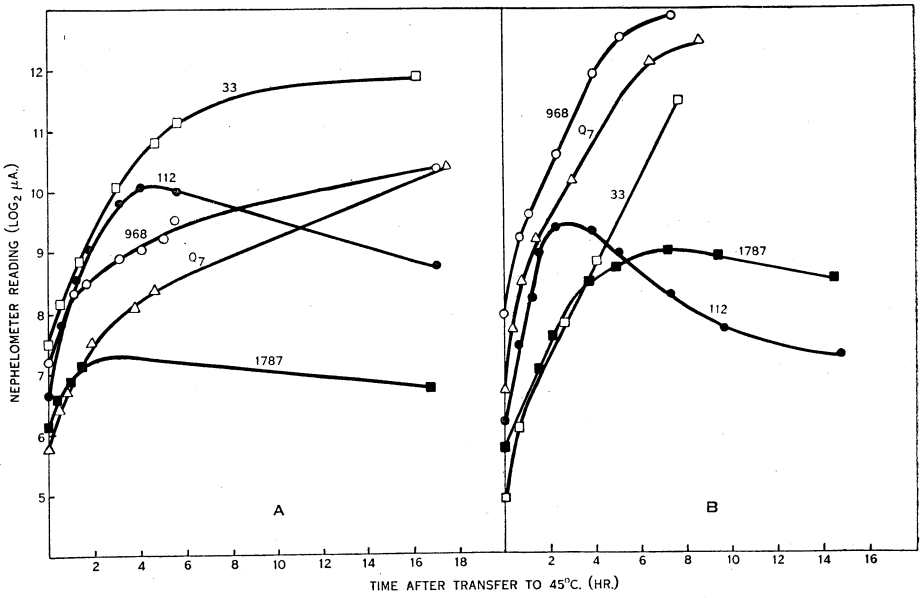

Fig. 5.—Growth of various strains at 45°C. after transfer from 37°C. *A*, N.Y.G. medium; *B*, P.D. medium.

Similar experiments have been performed by transferring cultures from 30 or 37°C. to 47.5°C. Some typical results are shown in Figure 6. At this temperature there is no continued growth on the N.Y.G. medium apart from the initial high rate maintained for 1-2 hr. On the P.D. medium most strains show no sustained growth beyond the first 2 hr. but some strains do continue to grow at rates varying between 0.2 and 0.3 approximately. At 47.5°C. and, to a lesser extent, at 45°C., gas production is much less marked than at lower temperatures.

When cultures were transferred to 50°C. there was no growth apart from a very small rise in turbidity during the first 30 min. Thereafter the readings remained constant or steadily fell.

Two additional experiments were performed in C.M.M. In the first of these nine strains (two type *A* and seven type *B*) were inoculated with the usual spore inoculum and after sealing under vacuum were incubated at 37°C. for 6 hr. This period was selected as sufficient to allow germination of spores and the beginning of vegetative growth. The cultures were then transferred to 50°C. for 7 days. After opening, 0.2 ml. aliquots were injected into mice and 0.5 ml. aliquots into duplicate tubes of pork infusion thioglycollate agar.

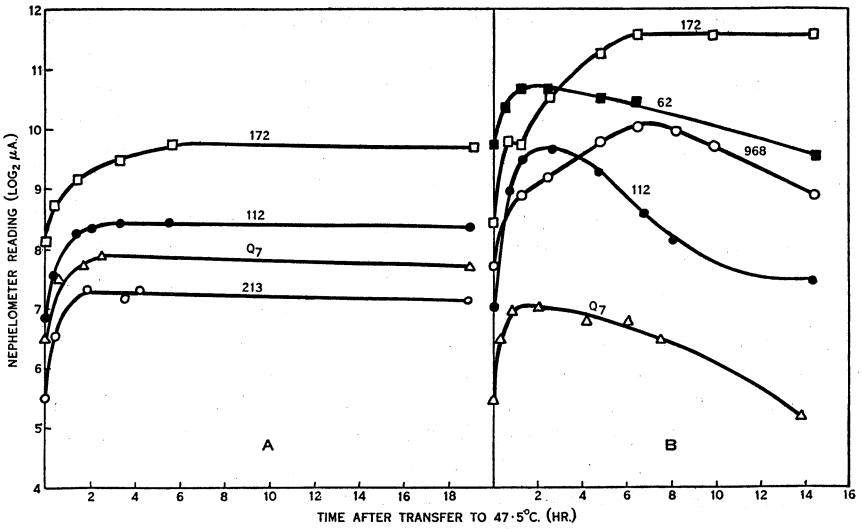

Fig. 6.—Growth of various strains at 47.5°C. after transfer from 37°C. *A*, N.Y.G. medium; *B*, P.D. medium.

All the mice remained well and free from symptoms of botulism. No viable organisms were detected in two cultures and in others the count, after 3 days at 37°C., varied from 2 to 45 per ml. The cultures showed no loss of vacuum during incubation at 50°C., no signs of digestion of the meat, nor was there any microscopic evidence that growth had occurred. The pH varied between 6.9 and 7.0. In the second experiment seven of the above nine strains (two *A* and five *B*) were inoculated into C.M.M., using 0.5 ml. ($> 10^8$ cells) of a C.M.M. culture grown for 20 hr. at 37°C. The cultures were held for 7 days at 50°C. Again there was no macroscopic or microscopic evidence of growth, and no toxin was detected. The pH was between 6.8 and 6.9. Viable counts were not done, but all cultures were returned to 37°C. for 5 days. Growth ensued in one culture only, the original inoculum in the remaining cultures having died during the 7 days at 50°C.

The data show that growth is adversely affected by a temperature of 45°C. and still more so at 47.5°C. although some strains in one medium still were capable of limited growth at the higher temperature. No evidence of growth was obtained at 50°C. and, indeed, it appears that vegetative inocula die at this temperature.

IV. Discussion

The present results confirm the report of Tanner and Oglesby (1936) that vegetative growth may proceed at temperatures somewhat lower than those permitting germination of spores. They also show that a similar situation exists in the upper temperature range, vegetative growth being possible at temperatures above the limits for spore germination. Trustworthy as these facts might appear it should, nevertheless, be recognized that very little is known about the basic conditions which determine the chances of successful germination of spores. For this reason it would be wise to place some reservations on the observations that spore germination did not occur at 12.5 or 45°C. Under different environmental conditions germination might occur at these temperatures.

When the range of temperatures over which vegetative growth occurs is considered, the present results are in good agreement with earlier workers regarding the lower limits, but there is some disagreement about the maximum temperatures. Although Tanner and Oglesby (1936) report some growth at 10°C. there was some uncertainty in their control of temperature which may have affected their results. The present experiments show a very large temperature coefficient which is increased as the temperature is lowered from 15 to 12.5°C. Even if, as is most unlikely, the temperature coefficient showed no further increase at lower temperatures the predicted rate of growth at 10°C. would correspond to one division in about 400 hr. This very low rate would be even lower if the temperature coefficient increased in the manner to be expected. It seems, therefore, safe to conclude that the probability of growth at 10°C. is very small. At the upper end of the temperature range the position is much less certain. Greer (1924) has reported growth of one strain each of type *A* and type *B* at 50 and 55°C. in beef heart medium under a vaseline seal. Apart from gas production the methods by which growth was assessed were not recorded, and no information is given on the accuracy with which temperatures were controlled. Meyer (1928), in a brief reference to his own observations, affirms that incubation at 55°C. produces involution forms and weak toxin but the experimental conditions under which these results were obtained are not given. The present experiments do not support the view that growth occurs at 55°C. and, indeed, have not yielded evidence of growth at 50°C. It is difficult to account for the discrepancy.

In the experiments now reported there can be little doubt about the reliability of the temperature control. Furthermore, there is clear evidence that in the media used growth at 45°C. was more irregular and slower than at 37°C. At 47.5°C. growth was even more restricted and, on one medium, was not continued beyond a period of about 2 hr. after transfer to this temperature. The trends in the temperature coefficient shown in Figure 1 substantiate the view that sustained growth on N.Y.G. medium is extremely unlikely at 50°C.

The evidence that growth at 45 and 47.5°C. is rather better on P.D. medium deserves some comment. There is no information which would show whether the improved performance on this medium is due to more efficient synthetic processes, or to a reduced rate of destruction of essential enzymes or metabolites. For this reason one is unable to reach any worth-while conclusions regarding

the probabilities of growth at still higher temperatures under different environmental conditions. The possibility of growth at somewhat higher temperatures is, however, worthy of serious consideration in the light of the effects of the ionic environment and of increased hydrostatic pressure on the thermostability of some enzymes.

All the strains examined showed their greatest rates of growth around 37-40°C. and the greatest yield of cells at similar temperatures. There is, therefore, no support for the view of many of the early workers that some strains had optimum temperatures less than 30°C. Many of these reports were based on estimates of the toxicity and it is most probable that the results were really a measure of other factors which are now known to affect the stability of the toxins. The strains used in these experiments have been isolated from widely different localities over a period of many years and may, therefore, be considered as representative. A point of some importance is that no large differences in the temperature requirements of the strains were detected, and no consistent differences between the type *A* and *B* strains could be demonstrated. Some differences in the temperature relations of the different strains do undoubtedly exist, but it is believed that these are relatively minor and that the emphasis should more properly be placed on the relative homogeneity of the group in their reactions to temperature. For this reason one would not expect the reactions of other type *A* and *B* strains to differ substantially from those described in this paper. It should, however, not be concluded that other toxigenic types of *Cl. botulinum* would also show similar behaviour. In this respect it is pertinent to draw attention to the observation by Dolman *et al.* (1950) that a type *E* strain grew and produced toxin at 6°C. This finding has been confirmed with all type *E* strains tested and the results will be published in a later paper.

V. References

Dolman, C. E., Chang, Helen, Kerr, Donna E., and Shearer, A. R. (1950).—Fish-borne and type E botulism: Two cases due to home pickled herring. *Canad. J. Publ. Hlth.* **41**: 215-29.

Dubovsky, B. J., and Meyer, K. F. (1922).—An experimental study of the methods available for the enrichment, demonstration and isolation of *Bac. botulinus* in specimens of soil and its products, in suspected food, in clinical and in necropsy material. *J. Infect. Dis.* **31**: 501-40.

Greer, F. (1924).—Growth of spore forming anaerobes at 50°C. *J. Bact.* **12**: 243-5.

Haines, R. B., and Elliott, E. M. L. (1944).—Some bacteriological aspects of dehydrated foods. *J. Hyg.* **43**: 370-81.

Mason, M. M. (1935).—A comparison of the maximal growth rates of various bacteria under optimal conditions. *J. Bact.* **29**: 103-10.

Meyer, K. F. (1928).—Handbuch der pathogenen Microorganismen (Kolle, Kraus u. Uhlenhuth) **4**: Botulismus 1269-1364.

Meyer, K. F., and Gunnison, J. B. (1929).—European strains of *Clostridium botulinum*. *J. Infect. Dis.* **45**: 96-105.

Monod, J. (1949).—The growth of bacterial cultures. *Annu. Rev. Microbiol.* **3**: 371-94.

Tanner, F. W., and Oglesby, E. W. (1936).—Influence of temperature on growth and toxin production of *Clostridium botulinum*. *Food Res.* **1**: 481-94.

Wagner, E., Meyer, K. F., and Dozier, C. C. (1925).—Studies on the metabolism of *B. botulinus* in various media. *J. Bact.* **10**: 321-412.