Table 1. Influence of auxin on flowering of Brussels sprouts when applied to plants of various ages. Ages at the beginning of cold treatment are given.

Age (wk)	Plants flowering (%)					
	Auxin before induction (ppm)		Auxin during induction (ppm)		Auxin after induction (ppm)	
	0	50	0	50	0	50
15	100	100	100	100	100	100
13	100	100	100	100	100	80
11	70	70	60	80	80	40
9	0	0	0	60	10	0
7	0	0	0	0	0	0
5	0	0	0	0	0	0

Six groups of plants of different ages, respectively 5, 7, 9, 11, 13, and 15 weeks old, were simultaneously given a cold treatment (5°C) of 9 weeks duration. To each group, auxin (0, 50, or 500 parts per million naphthaleneacetic acid in lanolin) was applied over a period of 10 days either before, during, or after the cold treatment to the debladed petiole stumps of the 2 youngest mature leaves. Each treatment was given to 10 plants. The plants were grown during the whole experiment (September 1954 to April 1955) in the greenhouse.

The percentages of plants that flowered in response to the cold treatment are given in Table 1. Just as expected, control plants younger than 11 weeks at the commencement of cold treatment did not flower except for one plant in the third series, which flowered at 9 weeks of age. However, plants 9 weeks of age treated with 50 parts per million of auxin during the cold treatment flowered in 60 percent of the cases. The auxin applications before or after the cold treatments were essentially ineffective in shortening the juvenile phase.

It is interesting to note that Stokes and Verkerk (*1*) have reported that earliest flowering occurs in this species at about the thirtieth node. Our data agree in that the average node of first flower of the 11-week-old plants was 31.8. However, the induced 9-week-old plants flowered at 19.3 nodes.

Not only did auxin paste treatment bring about flowering of apparently juvenile plants, and at a node lower than normal, but the 50-parts-per-million treatment also hastened floral initiation *in time* when it was applied during the cold treatment. The effects on time of flowering are shown in Fig. 1. Auxin applied during the cold treatment hastened the appearance of flower buds by 3 to 4 days. There was little effect resulting from treatment before the cold, and no effect with the aftertreatment. The effect of the high auxin application (500 parts per million) was much less pronounced. The question arises whether a lower concentration than the ones that were used would be still more effective.

The lengths of flower stalks yielded an interestingly similar response, as shown in Table 2. It can be seen that stalk elongation was increased by auxin applied during the cold induction. This increase ranged from 16 to 52 percent. Auxin applied before or after the induction had no apparent effect on length of flower stalks.

The data presented here indicate that under the test circumstances, auxin applications during cold treatment can permit floral initiation of otherwise juvenile plants of Brussels sprouts, at an unusually low node, with an acceleration evident both in the 9- and 11-week-old plants, and with a resultant increase in length of the flower stalks as well.

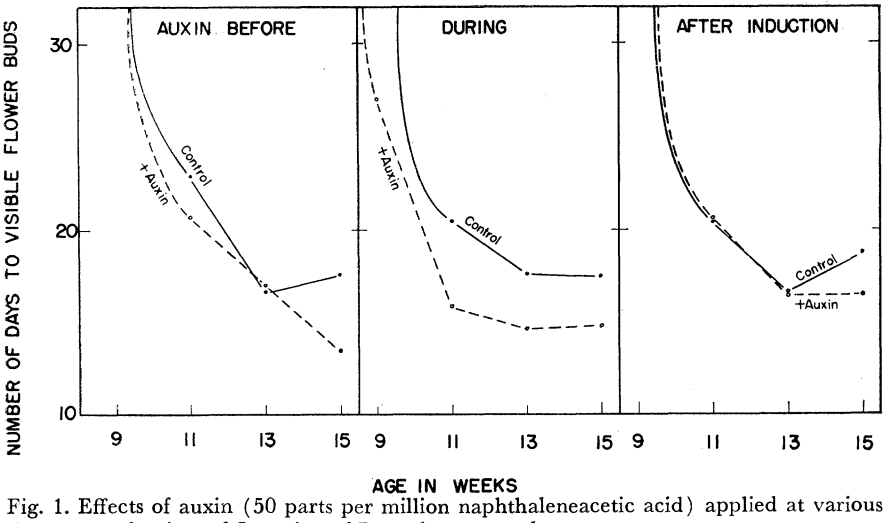

Fig. 1. Effects of auxin (50 parts per million naphthaleneacetic acid) applied at various times upon the time of flowering of Brussels sprouts plants.

Table 2. Influence of auxin applied during induction on the length of the flower stalk of Brussels sprouts. Ages of plants at the beginning of cold treatment are given.

Age (wk)	Length of flower stalk (cm)		
	Auxin concentration (ppm)		
	0	50	500
15	46.0	51.5	40.0
13	34.6	50.2	43.0
11	26.0	34.3	26.6
9		5.2	4.7
7			
5			

The results of these experiments extend the growing body of evidence that auxin treatments associated with low temperatures are promotive of flowering. Previous studies have reported such auxin promotions for species sensitive to photoperiods, to vernalization, and for indeterminant species (*8*). In the present experiments, auxin treatment resulted in flowering of otherwise juvenile plants. It is suggestive that the completion of the juvenile phase may be in part the accumulation of a sufficient auxin level at the apical meristem to bring about the condition receptive to cold.

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9 July 1955

Production of Toxin by Resting Cells of Cl. Parabotulinum Type A

The toxin of *Cl. parabotulinum* type A is a high molecular (*1*) protein distinguished by its extremely high biological potency (*2*). It appeared therefore that this system might provide an interesting model for a study of protein biosynthesis. Toxin formation by these cells is a rela-

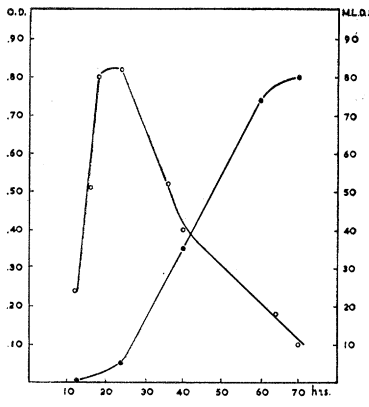

Fig. 1. Growth and toxin production by *Cl. parabotulinum* type A. ● —— ● Toxin titer (MLD × 10^4/ml); ○ —— ○ optical density (outside diameter) at 500 mμ, measured in the Coleman Junior Spectrophotometer.

tively rapid process, lending itself to a kinetic approach.

The growth curve of *Cl. parabotulinum* in a suitable medium is characterized by a sharp peak (between 18 and 24 hours), followed by rapid decline owing to autolysis. During the logarithmic phase little toxin is found in the medium. On continued incubation, the toxin titer increases steeply and reaches a maximum at the completion of lysis (Fig. 1). This curve suggested the possibility that the toxin is formed intracellularly and is released into the medium, upon autolysis.

In order to test this hypothesis and to determine the essential factors governing toxin production in the absence of growth of the organism, the technique of resting cells was employed.

Cl. parabotulinum type A (strain 2) was grown in a semisynthetic medium (*3*). After incubation for 18 hours, the cells were collected by centrifugation and were washed with saline containing 0.05 percent thioglycolate or with fresh medium as desired. The cells were then taken up in a small amount of thioglycolate-saline and added to the reaction mixture; the final cell concentration, as measured by optical density, was 3 to 4 times higher than that of the original culture. Incubation was carried out at 34°C in evacuated jars; subsequent experiments under aerobic conditions gave the same results.

Table 1. Toxin production by nonproliferating cells (thick suspensions) of *Cl. parabotulinum* type A

Omissions from complete medium*	MLD/ml
None	2,500,000
Vitamins	2,500,000
Amino acids	100,000
9 nonessential amino acids	2,200,000
Tryptophan	150,000
Glucose	200,000
Salts A (phosphates)	300,000
Salts B (NaCl, $MgSO_4$, $MnCl_2$, $FeSO_4$)	1,750,000
None; addition of penicillin (1000 units/ml)	2,500,000
None; addition of α,α-dipyridyl (50 μg/ml)	2,000,000

* Complete culture medium: synthetic medium as described previously (*3*). Toxin titer at zero time: 10,000 MLD/ml. Incubation period: 24 hours at 34°C.

At the start of the experiment and after incubation for 24 hours, the titer of the toxin was determined by intraperitoneal injection into mice, using the MLD procedure (*4*). As is shown in Table 1, the omission of either salt solution B, vitamins, or nonessential amino acids from the complete medium did not affect appreciably toxin formation. On the other hand, the absence of glucose, phosphates (salt solution A), or even one of the amino acids essential for growth, for example, tryptophan (*3*), resulted in a drastic reduction of the toxin yield. The fact that glucose is required for toxin production but not for growth suggests that a rich source of energy is necessary for the synthesis of the toxin.

The observation that certain nutrilites are required, both for growth and for toxin production, made it necessary to prove more stringently that under the conditions employed the formation of the toxic protein took place in the absence of cell multiplication. Such a proof was provided by the finding that penicillin (1000 to 10,000 units/ml) and α,α-dipyridyl (50 μg/ml) did not affect toxin production at all, whereas they suppressed growth completely even at lower concentrations (1 unit/ml or 20 μg/ml, respectively) (Table 1).

Before measurable lysis took place, only small amounts of toxin (20,000 to 100,000 MLD/ml) could be demonstrated in the cell suspensions. When, however, lysis was induced artificially by sonic oscillations (exposure for 15 minutes to vibrations in the 9KC Raytheon oscillator), large quantities of toxin were released into the medium. These experiments provide, therefore, direct evidence for the intracellular synthesis of the botulinus toxin. In addition it must be concluded that the toxin present *within* the cell is not detectable by the *in vivo* test employed.

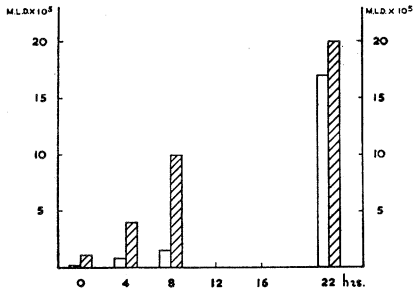

Fig. 2. Rate of toxin formation (free and total) by resting cells of *Cl. parabotulinum* type A. Unshaded bars, free toxin; shaded bars, total toxin (obtained by sonic treatment). Resting cells of *Cl. parabotulinum* type A, suspended in complete medium (see Table 1) and incubated at 34°C.

By using the sonic treatment, the amounts of toxin formed intracellularly after various periods of time could be determined. The results as illustrated in Fig. 2 appear to warrant the following conclusions: (i) Comparatively small amounts of toxin are produced by growing cells up to the end of the logarithmic phase (50,000 to 100,000 MLD/ml as assayed after release of the toxin by sonic disruption). (ii) Resting cells (in a medium containing the necessary ingredients) produce toxin at a very rapid rate; an eightfold increase is reached within 4 hours, thus giving about 40 percent of the total amount formed after 24 hours.

The procedure described offers a convenient system for a study of the biosynthesis of the botulinus toxin in the absence of cell multiplication.

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4. MLD represents minimal lethal dose.

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Important principles may and must be flexible.—ABRAHAM LINCOLN.