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Preliminary Investigation of the Inhibitory Effect of Maltotriose on the Bacterium *Erwinia carotovora*

by

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PRELIMINARY INVESTIGATION OF THE INHIBITORY EFFECT OF MALTOTRIOSE ON THE BACTERIUM *Erwinia carotovora*

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T O M Y M O T H E R

To

Lilia

Jorge

Marcela

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A B S T R A C T

The inhibitory effects of maltotriose (3G) on Erwinia carotovora were explored both in-vitro and in-vivo. Complete inhibition was obtained at an inhibitor concentration of 5000 µgm/ml, when growth on a minimal medium was carried out at 4°C for 8 days. Under identical conditions except at 10° and 27°C, inhibition was only partial. Also, inhibition was obtained when 3G impregnated disks were placed upon potato slices inoculated with the test organism. Although maltotriose has the basic tri-glucosidic structure of many glucosidic antibiotics, it lacks reactive side groups that appear to confer specificity and reactivity upon different antibiotics. The detailed biochemical mechanism of inhibition by 3G is not known, gross morphological changes suggest interference with protein and perhaps membrane synthesis. The most readily observable effect of 3G on growing cells of E. carotovora was a large increase in cell length with cell width remaining constant. Since maltotriose is relatively common in our food supplies and it possesses a very narrow bacterial inhibitory spectrum, this suggests that it might have utility in the control of soft rot and black leg in horticultural materials.

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I N T R O D U C T I O N

A disease in a plant, has been defined by several investigators, but the definition by Whetzel (1935) seems to be the only one that serves as a logical basis for study of plant pathology (Robert and Boothroyd, 1975). Whetzel stated that a "disease in a plant consists of a series of harmful physiological processes caused by continuous irritation of the plant by a primary agent".

The classification of plant diseases has been based on several factors including: crops affected, organs attacked, the symptoms of disease, the source of inoculum and the physiology of the diseased plants. Classifications of this type have not been based on the taxonomy of pathogens because with a classification like this, the fact that a disease is a harmful physiological process is lost.

Causes of Plant Diseases by Microorganisms

1. Fungi

Of all the microorganisms that attack plants, fungi cause the most damage. Like all the other plant pathogens, they establish a parasitic relationship

with the plant, depending on this way, on food produced by green plants.

Spores of fungi are carried great distances by winds, falling on leaves or stems, followed by germination, and finally the establishment of a parasitic relationship. A single spore can infect a plant and produce in a little more than a week, one hundred thousand or more spores. They can survive the winter in soil and on stubble, causing new infections the following spring.

2. Bacteria

They cause disease of many crops, producing water soaked spots on the leaves of many kinds of plants, as well as wilts when they attack the roots. Bacteria are spread by winds, splashing rain and also by infected seeds. Like molds, some of these microorganisms can survive winter, reproducing the disease the following spring.

3. Viruses

A difficulty exists in the study of diseases caused by viruses, since it is still debatable as to whether or not viruses are true microorganisms. Although some viral diseases can be transmitted from plant to plant

just by direct contact, most viral diseases are transmitted by insects. Viruses can overwinter in perennial or biennial weeds and in some cases, inside seeds (McDonald, 1978).

Rot Pathogens

All rot pathogens initiate disease in the same general way. They produce extracellular enzymes that will start the degenerative process that will result in the death of the plant tissue. Since these organisms obtain their food from dead plant tissue, they are classified as facultative parasites.

The development of most rots is favored by moisture and warm temperatures. During storage, where the rots represent the biggest problem it is very important to control temperature and humidity conditions as well as controlling the store of susceptible plant parts to avoid the spread of the disease (Roberts and Boothroyd, 1975). Bacterial soft rot is universal, and it represents a large loss of vegetables and ornamental plants.

The causal agent of bacterial soft rot has been identified as Erwinia carotovora (Roberts and Boothroyd, 1975).

Attempts to control this organism have been done through the control of insects, careful handling, well ventilated storages, exclusion of disease vegetative plants from certain areas or erradication of the microorganism by crop rotation. Despite all efforts to control or solve the problem of soft rot caused by species of Erwinia, no practical solution has been found to date.

Recognizing the universal nature of Erwinia and the constant vigilance required to control bacterial contaminations, one avenue of possible benefit might be the use of inhibitors to block microbial growth. Any inhibitor would have to be highly selective for plant pathogens (especially Erwinia), non toxic to higher forms of life and readily available and economical to use. One possible compound that could satisfy all these requirements is maltotriose (3G) which was reported by Kondo et al., 1975, to have antimicrobial activity aginst Erwinia carotovra. These workers, from a pharmaceutical institute, were primarily concerned with the occurrence of 3G in fermentation broths, they did not pursue practical applications of such observations.

The purpose of this thesis was to confirm that 3G did inhibit Erwinia both in vitro and in vivo, to undertake a

preliminary characterization of any such inhibition and to consider a preliminary assessment of the practical significance of any observed inhibition.

LITERATURE REVIEW

All agricultural products contain certain microorganisms that constitute the normal flora found only on the exterior surface of plants. This surface acts as an effective barrier against microbial invasion and the interior tissue can be regarded as sterile. One of the consequences of improper handling of these products is spoilage due to microorganisms. When the superficial tissue of a vegetable or fruit is wounded for any reason, microorganisms can penetrate to internal parts causing breakdown of different structures and sometimes, the complete destruction of the product. A group of microorganisms that belong to this type is the genus Erwinia. This genus was proposed originally by a committee based on the idea that all peritrichous plant pathogens be grouped under a genus called Erwinia. Erwin Frink Smith, whose name was given to Erwinia, did not agree with this vague concept, but his advice was not taken and the genus remained as such.

Early in the twentieth century, several soft rot diseases of plants were studied, and the bacterial species which caused them were named in accordance with the host plant from which

they were isolated. Also, it was found that the soft rot bacteria, were different from Erwinia amylovora, the first proven causal agent of a plant disease. Many attempts have been made to revise the nomenclature of the genus. Martinec and Kocur (1963) recognized two species: E. carotovora and E. amylovora. Dye (1968) recognized five species: E. amylovora, E. herbicola, E. uredovorus, E. stewartii and E. carotovora. Lelliot (1974) based on acid production in peptone water sugars and a series of biochemical tests, placed the species in three groups: E. amylovora with eight varieties, E. herbicola with four varieties and E. carotovora with five varieties.

Many researchers still disagree with this classification, and studies have been made to clarify the nomenclature of the genus. These studies are based on morphological, physiological and biochemical characteristics. For example: based on fermentation of sugars, Katznelson (1955) found that Erwinia cultures were the only species that could anaerobically attack glucose. From similar studies by White and Starr (1971) it was concluded that:

1. Due to the same end product patterns, this genus could be a member of the family Enterobacteriaceae.

2. All the strains labelled E. carotovora, except for one strain, have the same fermentation end products (lactase, formate, succinate, acetate, 3 butanodiol ethanol and CO_2).
3. The fermentation patterns of E. amylovora are heterogeneous.
4. The experimental conditions for these results are critical and the effect of any factor is unknown in the metabolism of Erwinia.
5. The results from fermentation end products patterns alone are not sufficient to classify this genus.

Starr and Mendel (1969) concluded from G-C content tests, that certain groups of Erwinia can be grouped into differentiated clusters and that these clusters correlate with certain existing nomenclature groupings.

Other studies were undertaken to compare phenotypic properties from several hosts of E. chrysanthemi. Dickey (1979) tested several biochemical, morphological and physiological properties in various strains of E. chrysanthemi, E. carotovora, E. cypripedii and E. rhapontici. It was found that the phenotypic characteristics could be used for identification of E. chrysanthemi, but not for the distinction of E. carotovora and E. atroseptica. Lelliot

(1974) described these cells as predominantly single straight rods, 0.5 - 1.0 by 1.0 - 3.0 microns. All of them, except one, are motile by peritrichous flagella, and all are gram negative. Their optimun temperature for growth is between 27 - 30°C, they are facultative anaerobic and are always associated with plants as pathogens, saprophytes or constituents of the flora.

Erwinia carotovora

One of the species of the genus Erwinia is E. carotovora. This specie includes four varieties, the most common being carotovora and atroseptica. The first authentic description of a pathogen causing blackleg and soft rot was given by Van Hall (1902) who used the name of Bacillus atrosepticus for the causative organisms. One year before, Jones (1901) described a microorganism that produced soft rot in many fleshy vegetables and plant parts, which was named Bacillus carotovorus. Since that time, many studies have been done on plant pathogens causing rots. Several species have proven to be synonymous to species described by Jones (1901) and Van Hall (1902). However, none of the researchers completely agree as to whether these organisms were really

different. On the other hand, some researchers thought that the rots were caused by a group of coliforms, too heterogeneous for a definite classification.

Many tests have also been done to determine the differentiation of these two varieties. Tests include morphological, cultural, biochemical and pathogenic analyses. For example, Van Hall and Jones (Smith 1949) found definite differences between the two varieties in the following:

a) the stain of the flagella

b) cultural characteristics in certain media

c) maximum growth temperatures

d) carbon utilization.

Carbon utilization is considered one of the most important. Erwinia carotovora could utilize sodium salts of hippuric, malonic and uric acid, also erythrol and ethyl alcohol. Erwinia atroseptica did not, however, use these types of compounds. From such tests, it was concluded that the pathogens were two distinct species. Later, Dickey (1979) concluded from physiological and biochemical analyses, that the two varieties, could not be distinguished.

Apart from these types of analyses, pathogenic tests have been used to prove or disprove similarities of the

pathogen. Smith (1950), while working with potato plants, found that depending upon the site of inoculation of a plant, the amount of growth between the species was different. It was concluded that the two pathogens were distinct species. Also, it was noted that E. atroseptica caused soft rot and black leg, while E. carotovora did not cause black leg on growing plants. Some of the biochemical reactions of both varieties of Erwinia are listed in Table 1.

Hosts of Erwinia carotovora

Cultures of this microorganism have been isolated from banana, corn, cucumber, dieffenbachia, dracaena, iris, lettuce, poinsettia, tobacco, wax plant, zucchini, caladium carrot, potato, callo, cabbage, tomato, onion, chrysanthemum, celery, green pepper, etc. (Dickey, 1978). It has been detected in tubers, seed stocks and soils. The success of isolating this variety depends mainly on the media used. In Manitoba, E. carotovora has been isolated from soil, and its association with plant debris is known to prolong its life (Burr and Scroth, 1977; Poff, 1979).

Table 1

Some Biochemical Reactions of two Varieties of Erwinia carotovora

	E. carotovora var. carotovora	E. carotovora var. atroseptica
* -Methyl glucoside	-	+
* Xylose	+	+
* Lactose	+	+
* Melezitose	-	-
* Maltose	d	+
* Dextrin	-	-
* Glycerol	-	-
* Ribose	+	+
Anaerobic growth	+	+
H_2S from cysteine	+	+
Sucrose, reducing compounds	-	+
Pectate-degradation	+	+
Motility	+	+
Nitrate reduction	+	+
Gas from glucose	d	d
Lecithinase	-	-
Phosphatase	-	-
Sensitivity to erythromycin (50 μg)	-	-

* Acid production from organic compounds by Erwinia species and their varieties.
d 11 - 89% strains positive (Lelliot, 1974).

Soft Rot and Black Leg

Erwinia carotovora causes black leg and soft rot of potato, these are the cause of considerable loss in the field, transit and storage. Erwinia carotovora var. carotovora is the causal agent of soft rot while E. carotovora var. atroseptica causes black leg and a storage rot of potato tubers (Boothroyd, 1975).

The bacterial soft rot of plant tissue occurs all over the world. It starts when bacteria are deposited in wounds of susceptible plants, usually when the plant is harvested. Rots occur in roots, stems, leaves, etc. The E. carotovora produces extracellular pectolytic enzyme that digest the middle lamella. After reaching this stage, the cells are nourished by nutrients that exude from the wounded tissue. The bacterial cells multiply and attack healthy susceptible plant cells. The results of this maceration is the death of the plant cells, and the process continues until the plant part has rotted completely.

The bacteria ooze out of the diseased tissue and may be spread to the soil and other plants, they can also be spread by dipterous insects. The type of contamination by this vehicle has been found to be dependent on the time

of the season. For example, E.c. var. atroseptica is found more often early in the season, while E.c. var. carotovora increases later (Kloepper, Harrison and Bewer, 1979; Molina, Harrison and Bewer, 1974).

As soft rot, black leg appears as a water soaked lesion that gradually enlarges due to an increased activity of pectolytic enzymes. The characteristic symptom of black leg is the blackening of the basal part of affected stem. At flowering time, the symptoms are characterized by rolling and chlorosis of the upper leaves, and later the plants die, as the lower stem is surrounded with rot. As before, in this case bacteria are released into the soil and can contaminate new plants.

There are many factors that will affect the development of this disease, such as field washing and post harvest chilling (Segall, 1967). Also, temperature, relative humidity, host susceptibility etc. However, even if all the factors are favorable for the development of disease, if a minimum threshold pathogen population is not present, then the development of black leg will fail. For example, in Majestic potato cultivars, this threshold population of 10^8 bacteria/gm of stem was considered a prerequisite (Erinle,1975).

In the case of soft rot, it has been observed in potato storage, that the temperature, moisture and the age of the tubers are factors intimately associated with the development of the disease (Kendrick, Wedding and Paulus, 1955). It was concluded that in general, the higher the temperature and humidity, the more quickly the infection became apparent (Table 2).

Ventilation is another factor very important during the storage of vegetables, because it assists in maintaining a non-fluctuating temperature during the storage period. The needed air for this purpose will depend upon the tuber temperature at harvest time, amount of bruising, rate of respiration, speed of cooling, etc. However, the way the conditions can be obtained and maintained may differ from area to area. Excellent results have been achieved at 95% R.H., at an air movement rate of 18.7m3/h (Sparks, 1980).

In the case of potato, attempts to control soft rot and black leg have been tried by crop rotation, disease free stock, control of insects, avoidance of bruising during harvest. On the other hand, if tubers have been bruised, it is recommended to cover them with tarpaulins during transit between field and storage to increase relative

Table 2

Correlation of the Temperature - Relative Humidity Index with the Incidence of Bacterial Soft Rot of Potato Tubers

Infected Potatoes - %	Temperature - Humidity Index
1	7.2
2	6.8
3	7.8
3.05	8.0
9	8.5
14	8.9
15	9.8
25	9.0
28	10.4
32	10.6
50	13.1
51	11.3

humidity allowing suberization.

Storage is critical for the development of Erwinia carotovora, so potatoes should have the optimum conditions for suberization (which are 12 to 18°C and an RH of 90 - 95%) and good ventilation, then the temperature will be gradually reduced to 4°C. Under these conditions, the tubers will not sprout in storage. Storage parameters may differ according to the final usage of the tuber (McCollum, 1975; Anon., 1979).

Enzyme Production

Many plant pathogenic and non pathogenic microorganisms like Bacillus, Flavobacterium, Pseudomonas, Enterobacter, Clostridium, Erwinia, Xanthomonas, Yersinia and Klebsiella, have been known to produce pectinolytic enzymes (Chatterjee, 1978; Perombelon, 1979). Reed (1975) classified these enzymes as follows:

1. Pectic enzymes acting mainly on pectin:

   Polymethylgalacturonases (PMG) — endo, exo

   Pectin lyases (PL) — endo, exo

2. Pectic enzymes acting on pectic acid:

   Polygalacturonases (PG) — endo / exo

   Pectate lyases (PAL) or (PATE) — endo / exo

3. Pectinesterases.

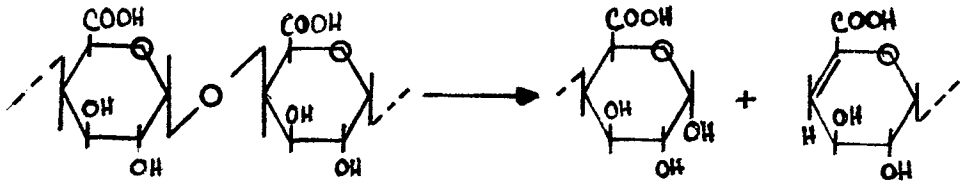
Some of the microorganisms discussed earlier have been tested for the production of certain pectolytic enzymes to determine the correlation between virulance and enzyme activity. It was shown by Knösel and Lange (1972) that after 24 hours, the media where these microorganisms were incubated, contained breakdown products of pectate. It was concluded that under acid conditions, pectate was degraded by endo-polygalacturonases by hydrolytic reaction, while endo pectic acid transeliminases produced degradation in alkaline conditions. When experiments to cause infection were done, suspensions of 10^4 to 10^6 cells/ml were used. The results showed chlorotic spots, lesions, rotting and necroses. It was demonstrated that the relation between virulance and enzyme activity was different for each microorganism.

Pectate Lyases

These types of enzymes have been isolated from various bacilli, with Bacillus polymyxa lyase the representative enzyme (Reed, 1975). Erwinia carotovora produces a similar enzyme, whose favorite substrate is polygalacturonic acid. Moran et. al. (1968) reported that the extracellular polygalacturonic acid transeliminase (PATE) from E. carotovora had an optimum pH of 8.5, a maximum activity at 50°C and was activated by $CaCl_2$. The purified enzyme degraded polygalacturonic acid, but there was no action upon pectin. The higher oligouronides, penta, tetra and trigalacturonic acid, predominated in the early stages of the reaction, while digalacturonic acid was the major component of the end products.

Intracellular PATE was also found to degrade polygalacturonic acid. Its optimum pH was 8.5 and it was also activated with $CaCl_2$. It was observed that the rate of degradation was proportional to the chain length of the substrate. As indicated before, the presence of higher oligogalacturonides in the initial stages of the reaction, indicates the possibility of an endo-PATE rather than an exo-PATE. Also, it was concluded that the degradation was

a transelimination reaction due to the double bond between C_4 and C_5:



Finally, these researchers concluded that both enzymes are probably identical since they both have the same characteristics and end products.

Since PATE is probably the same exo and intracellular enzyme, Shinji (1977) did investigate the induction of these enzymes. It was found that the non-induced PATE activity was three to four times greater in cell-free extracts than in the culture filtrate during the log phase. After induction, the intracellular enzyme activity was found to be 130 times higher. To induce the formation of the enzyme, pectic acid was tested. The utilization of this acid resulted in a long lag period, suggesting that this was not the true inducing metabolite. The major end product of the cleavage of pectic acid by E. carotovora was unsaturated digalacturonic acid (UDG). This compound produced a rapid induction, but it is still unknown as to whether or not this compound is the true inducer. Shinji (1977)

suggested that the control of the product - induction could be a mechanism by which enzymes could be regulated.

The pectolytic activity of phytopathogenic enterobacteria was, until recently, the only known degradative activity of this type. Chatterjee et.al. (1979) found that Klebsiella and Yersinia, which are not phytopathogenic, possess polygalacturonic acid transeliminase (PATE), and often hydrolytic polygalacturonase (PG). After being unable to detect maceration of plant tissue by the pectolytic enzymes of these microorganisms, they concluded that in comparison to these enzymes, which just present a catabolic function, the enzymes of Erwinia have a cytolytic as well as a catabolic function. In addition, the enzymes in Erwinia are produced in greater quantities than in the other two microorganisms.

When testing different carbon sources for the production of PATE, it was found that in the case of PATE from Erwinia, although the polygalacturonic acid was the most effective substrate for induction of activity, the ratio of specific activities was higher with other carbon sources. It would appear then, that a portion of the total activity remains cell-bound.

A study by Almengor-Hecht and Bull (1978) investigated chemostat techniques for the enrichment isolation of extracellular enzyme-producing microorganisms. They found that when using limited carbon media containing a highly methylated pectin, in a natural mixed microflora, a pectinolytic dominant microorganism developed. This microorganism was a gram negative rod, motile by peritrichous flagella and on the basis of physiological and biochemical tests, this bacterium was identified as Erwinia carotovora. The pectinolytic activity was not due to a pectin esterase but was due to a pectin lyase. Also, they concluded that due to the final presence of only unsaturated monomers and dimers, as well as the rate of reduction in viscosity of pectins, this lyase was probably an exolyase type. It was suggested that this technique be used for the isolation of microorganisms having defined properties, especially for those organisms that produce an extracellular enzyme.

Perombelon et al. (1979) working with Clostridium and Erwinia, found that another way of differentiating microorganisms was by studying the relationship between size of the decayed pectinolytic area, of potato for example, and the incubation

temperature. It was observed that at 20°C, Clostridia were responsible for most of the rotting, while at 16°C, E. carotovora greatly accelerate the onset of rotting.

Pectin Methylesterases

These types of enzymes are also found in bacteria like soft rot Erwinia or Xanthomonas. They hydrolyze the methyl ester of galacturonic acid. In bacteria, they have pH optima on the alkaline side (7.5 - 8.0), and little is known about their synthesis and excretion.

The pectinases of Erwinia carotovora not only produce rots of plants but have been observed to produce other reactions. Lovrekovich et al. (1967) inoculated potatoes with 10^{10} cells/ml of Erwinia carotovora and after 24 hours of incubation at room temperature, the inoculation pore was surrounded by a large white zone of rotted tissue and the zone ended with a narrow black ring. After several tests, it was concluded that the amount of rotted tissue was influenced by the amount of bacteria used for inoculation, the time between cutting of the tuber and inoculation plus the amount of moisture.

When the white zone was analyzed, it was found to have

a very high dehydrogenase activity in comparison to the healthy tissue. If the cells were removed from the rotted tissue by centrifugation, the supernatant showed no activity, suggesting that the dehydrogenase activity may be from the bacteria. This activity inhibited phenol oxidation, which is the cause of the dark color.

When the black ring was analyzed, it was found that the zone was constituted by macerated tissue, suggesting an activity of a bacterial pectinase. To verify this, healthy tubers were inoculated with commercial pectinase, and after incubation and development of the same symptoms, the researchers concluded that the black ring may be induced by pectinase diffusing out of the inner tissue that contains the bacteria. As the bacteria could not be isolated from the black ring, it was suggested that this zone was involved in the defense mechanism of the plant.

Based on these results, Lovrekovich et.al. (1967) proposed a theory for the development of the disease symptoms. They suggested that after incubation of the potato, the rapid increase of the microorganism was accompanied by production of pectic enzymes by bacteria. These enzymes diffused across the tissue inducing the formation of oxidases and therefore,

the oxidation of phenols. As pectic enzymes diffused faster than the bacteria, the induction of the oxidation of phenols became evident by the formation of the black ring, which constituted a defense barrier. Inside the black ring, the bacterial dehydrogenase activity, acted by inhibiting the oxidation of phenols, and therefore, the defense barrier is not formed in this area.

Another characteristic of the pectinases of Erwinia, is a synergistic effect. When PME and transeliminases of E. carotovora and P. cryptogea were inoculated in a summer squash, more enzymes were noticed in the extracts, rather than in extracts of fruits inoculated with either microorganisms. The activity of these enzymes was more than the double activity of both E. carotovora and P. cryptogea when grown or inoculated separately (El-Goorain, 1976).

Inhibition and Antibiotics

Several unsuccessful attempts have been made to control soft rot produced by Erwinia carotovora. For example, when control atmospheres were tested, it was found that concentrations of CO_2 above 10% were necessary for inhibition of Erwinia. Unfortunately, this level is not recommended

for the storage of most vegetables and fruits (Wells, 1974). Significant reductions of bacterial growth were also obtained when the concentrations of O_2 were lowered between 1 and 3%. This range is well tolerated in many agricultural commodities during storage. In contrast, when the concentration of CO_2 was decreased to very low levels, a decrease in the growth of Erwinia was observed. At 0% CO_2 growth was completely inhibited. During these experiments, only CO_2 and O_2 were tested, so the effect of all the other air components appears to remain unknown.

Another example of preventing the growth of Erwinia was demonstrated by using pesticides like chloropheninphos, carbaryl and propoxur. When these insecticides were tested on species of Azotobacter, Agrobacterium, Arthinobacter, Bacillus, Brevibacterium, Erwinia, Micrococcus and Sarcina, an inhibition of the phosphatases and dehydrogenases of the respiration cycle was observed, as well as a disturbance of the catalytic function of catalases (Pawlaczyk, 1978). Also, it was observed that the phosphatase activity was affected causing a potential hazard to the phosphorus regime of water reservoirs.

Other attempts to control the soft rot microorganisms

have been done by crop rotation, which prevents a build up in the soil of the organisms, or sanitation of potato storages. In addition to these preventive methods, it is also important that planting is not done in soil that is sufficiently warm for growth. Also, bruised vegetables should be avoided, since wounds are the main entrance of microorganisms. If, for example, a bruise should occur in potatoes, it is very important to cover the tubers with tarpaulins (Anon., 1979). This will increase the relative humidity, reduce dehydration and allow suberization. Apart from these types of controls, many antibiotics have been tested with Erwinia. For example, when Erwinia was isolated from a case of conjuntivitis in a six month old child or when a second strain was isolated from the urine of a 51 year old woman who suffered a febrile disease, Sanchis-Bayarri (1975), confirmed the nature of the microorganism by a series of biochemical tests. When an antibiogram was made, it was observed that Erwinia had a sensitivity to ampicillin, furantoin, nalidixic acid, gentamicin, streptomycin and cephalothin.

Brazda (1976) studied the influence of chloramphenicol and streptomycin sulfate on Erwinia carotovora in-vitro and

in potato tubers. It was shown that sensitivity depended upon the strain and that the success for controlling the soft rot, was determined by the period between inoculations and the treatment of the tubers.

Lobanok (1977) tested the sensitivity of 788 strains of Erwinia isolated from natural substrates. The tests were done with respect to penicillin, streptomycin, tetracline, chloramphenicol and kanamycin. It was found that the 43.99% were drug resistant. Penicillin resistance was the most frequent. From the analysis of drug resistance spectra, it was shown that Erwinia strains resistant to one (172 strains) or two (149 strains) antiobiotics were the most frequent. Twenty five strains were multiresistant. In another experiment with antibiotics, Robinson (1979) tested 160 strains of gram negative bacteria, isolated from samples of vegetables sold in supermarkets and from samples of salads prepared in restaurants, hospitals and homes. When the microorganisms were identified, Robinson noticed a predominance of Pseudomonas and Erwinia. The resistance to ampicillin, cephalothin and carbenicillin presented by all 160 strains of isolated bacteria occurred in 108, 108 and 90 strains, respectively.

Another compound, other than an antibiotic, has been tested for the inhibition of Erwinia carotovora. This tetrasaccharide was isolated from cultures of streptomyces strains and it was proven to have an antimicrobial activity against E. carotovora. Kondo et al. (1974) tested 159 strains of streptomyces for this purpose, and it was observed that 26 strains showed the inhibitory effect just against gram negative bacteria, while three strains showed the same effect against other gram negative and positive bacteria. All of these results were obtained by the cylinder agar plate method. Inhibitory zones of 21.0 and 16.5 mm of diameter were found at concentrations of 3000 mcg/ml and 750 mcg/ml, respectively.

Based on physical chemical tests, the inhibitory agent was a saccharide. This compound was soluble in water, methanol and sparingly soluble in ethanol, iso-propanol, n-butanol and iso butanol. It was insoluble in ether, chloroform, benzene, ethylacetate and acetone. When the antibiotic was hydrolyzed with 0.5 M HCL, and tested on thin layer chromatography, the end product of hydrolysis was confirmed as glucose. The original product was proven to be maltotetraose by a silica gel thin layer chromatography.

Other antimicrobial maltooligosaccharides were tested against E. carotovora (Kondo et al., 1974) and the results are as follows:

Maltooligosaccharide	Relative activity %
Maltose	8
Maltotriose	105
Maltotetraose	100
Maltopentaose	75
Maltohexaose	40
Glucose	0

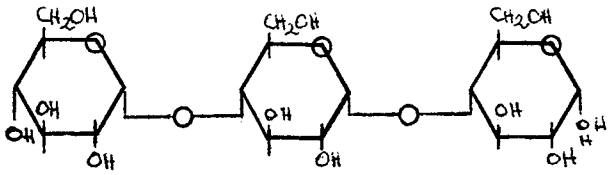
Tanaka (1975) found the same effect in 23 out of 159 streptomyces strains, and no activity was found when other gram positive and negative bacteria were used. It was shown that the antibiotic was produced by enzymatic activity of the streptomyces amylase. This enzyme was found to hydrolyze starch to maltooligosaccharides and its activity was expressed as maltotetraose activity (mg/ml) against E. carotovora. The inhibitory zones found in this experiment were exactly the same as the ones found by Kondo et al. (1974). It was also observed that when a higher concentration of the enzyme was used, the concentration of maltose increased, while maltotriose and maltotetraose decreased, indicating more

complete hydrolysis, reduced inhibitory activity.

The enzyme was considered to be an ∝ amylase, which attacked the (1-4) and (1-6) linkages of starch at random, producing short chains called dextrins. The streptomyce amylase was found to have an optimum pH of 7.0 and an optimum temperature of 40°C.

Maltotriose, a trisaccharide, was shown to have antibiotic properties similar to maltotetraose (Kondo et al., 1974). Its chemical structure is as follows:

0 - ∝ - D Glucopyranosyl - (1-4) - 0∝D glucopyranosyl - (1-4) D glucose.

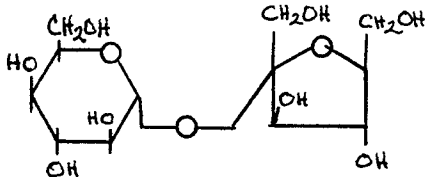


There are different ways to obtain this trisaccharide, for example:

a) From corn syrup. Depending upon the degree of hydrolysis, relative concentrations of dextrins, higher sugars, disaccharides and monosaccharides are found. Glucose and maltose are found approximately in a 42% concentration while maltotriose and maltotetraose are each found at approximately 20%. The rest of the dextrins represent

37% of the total solids (Oser, 1955).

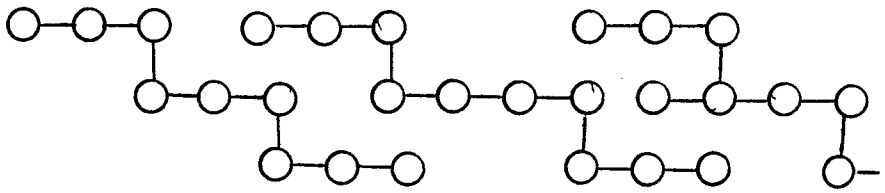
b) From sucrose. It is well known that sucrose is a double glycoside, being both β-D-fructofuranosyl and ∝-D-glucopyranoside and ∝-D glucopyranosil β-D fructofuranoside.



Therefore, sucrose can be hydrolyzed by either a β-D fructofuranosidase or an ∝-D glucopyranosidase. Saccharomyces cereviceae contains these types of enzymes, and at pH 6.9, it can hydrolyze sucrose as well as the resulting products of this hydrolyses, that is maltose, melezitose, ∝-∝trehalose and maltotriose which constitute 15 to 20% of the fermentable carbohydrates of brewers wort (Barnett, 1976).

c) From Pullulan. Pullulan is an extracellular glucan produced by fungus Pullularia pullulans by growth on sucrose as a carbon source. Partial hydrolysis of the glucan indicated that it contained 1 - 4 and 1 - 6 ∝ glucosidic linkages (Marshall, 1974).

When the glucan is treated with pullulanase, an enzyme produced from Aerobacter aerogenes, it was shown that no products containing 1 - 6 ∝-D-glucosidic linkages were found. It was also observed that when pullulan was treated with acid or high temperature, the inactivation of an associated ∝-D glucosidase was achieved, leaving maltotriose as the main product. When Marshall (1974) made a more careful examination of the products and the action of pullulanase, the presence of end products other than maltotriose was noted. Three possible chemical structures were suggested for the glucan, these were:



It is evident that the kind of enzyme with which pullulan is treated, will result in a different end product.

d) From waxy corn starch. An amylase from Streptomyces griseus, has the activity of producing maltotriose from starch. The trisaccharide was produced at a concentration of 51% suggesting that the amylase

cleaved regularly the third glucosidic bond from the non-reducing end of the substrates. The amylase showed a maximal activity at 45°C and pH 5.6 - 6.0.

Maltotriose Degradation

The product of maltotriose degradation by an Aspergillus oryzae amylase has been demonstrated to depend on the concentration of maltotriose. Allen and Thoma (1978), working with a reduced-end labelled maltotriose, found that at low concentration the only significant labelled product was glucose. As the concentration of maltotriose was raised, maltose became evident. At high concentration, 80% of the labelled product corresponded to maltose, suggesting the participation of more than one substrate molecule in the degradation of maltotriose at high concentration.

It has been considered by Allen and Thoma (1978) that there are at least three multimolecular mechanisms:

1. Condensation: Polymerization of two molecules to form a new glucosidic bond that undergoes hydrolysis to smaller sugars. This type of reaction has been observed with carbohydrases such as α and β amylases as well as glucoamylase.

2. Shift two bindings. The binding site of a polymerase is composed of subsites that are complementary to and bind glucosyl units, allowing two molecules to bind simultaneously to one enzyme. Depending on the kind of binding, the second substrate molecule may or may not shift the first substrate exposing a different bond to cleavage. Not only does this type of change of substrate occur, but also the opposite one, where the second substrate causes a shift of the first one. This phenomenon can be explained by a transglycosilation mechanism, but it remains to be proven whether the second type of shift can occur.
3. Transglycosylation. This is the transfer of a glucosyl group from a donor to an acceptor, other than water. Based on kinetic studies, these researchers proposed a theory, where the enzyme reacts through an enzyme glucosyl intermediate form, from the non-reducing end of the substrate molecule. Allen and Thoma (1978) suggested that their theory can be applied for explaining the action of carbohydrases.

In human beings, the digestion of maltotriose was studied by Messer and Kerry (1967). It was observed that

the digestion of starch begins with the action of amylase found in the saliva. It continues in the stomach and then in the small intestine. From the action of the salivary amylase on starch, the main products were maltose, maltotriose and α-limit dextrins. The maltotriose constituted 37% from amylase and 28% from amylopectin. The action of duodenal juice on starch resulted in a maltotriose concentration of 25%, but the researchers suggested a higher concentration because the α limit dextrins are a source of maltotriose.

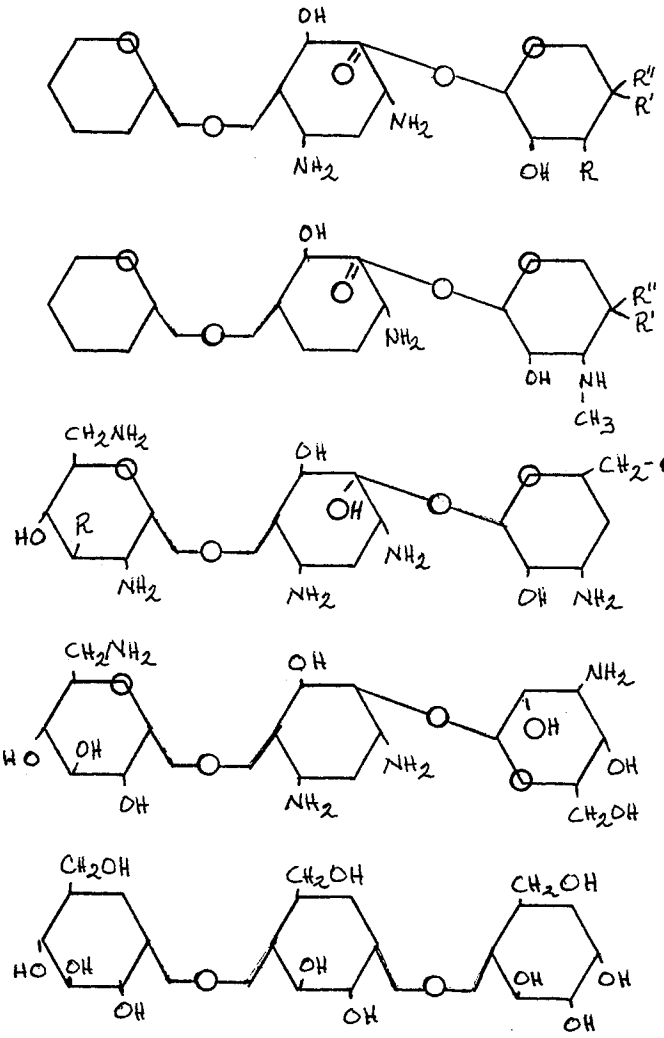
For the specific digestion of maltotriose, the human amylase from saliva, can catalyze this hydrolyses but the amount required is much greater than that for the digestion of starch. However, the intestinal mucosa is capable of digesting the trisaccharide, as well as maltose, making it apparent that the site of digestion is the small intestine mucosa.

The maltase activity of human small intestine can be divided into four fractions, based on heat inactivation. Maltase Iá is inactivated at 45^{o}C, maltase Ib at 50^{o}C, maltase II at 60^{o}C and maltase III at 75^{o}C. This difference was the main characteristic that led Messer and Kerry

(1967) to suggest that the maltotriase activity of human intestinal mucosa was due to three enzymes, and not to a specific maltotriase.

The chemical structure of maltotriose resembles the chemical structure of certain amynoglucoside antibiotics (Fig. I). Some of these compounds like streptomycin, kanamycin, paromomycin, neomycin, gentamycin and hygromycin B produce a disturbance of protein synthesis. This disturbance is the result of codon misreading, which is the result of an increased incorporation of certain kinds of amino acids into polypeptides (Tanaka, 1967). Also, Tanaka (1967) investigated the activity of the degradation products of kanamycin. It was observed that the antibiotic consisted of three moieties, these being: deoxy streptamine, 3 amino 3 deoxyglucose and 6 amino 6 deoxyglucose. Of the three molecules, only the first stimulated both polyribonucleotide and DNA by direct incorporation of amino acids into polypeptides. This molecule is found in kanamycin, neomycin, paromomycin, gentamycin, hygromycin B and streptomycin, but it is not a component of kasugamycin and spectinomycin. These last two compounds are also part of the amynoglucosides antibiotics, but their method of protein inhibition is

Figure 1. Chemical Structures of Certain Aminoglucoside Antibiotics



Gentamycin

Sisomisin

Nebramycin

Kanamycin

Maltotriose

different. These antibiotics contain a compound namely, N, N' methylactinamine which is a stereoisomer with streptamine. Tanaka suggested that deoxystreptamine plays an important role in misreading, during the synthesis of proteins.

It has been observed by Okamoto et al.(1965), that a derivative of streptomycin, dihydroestreptomycin, and kanamycin can be inactivated by enzymes from drug-resistant microorganisms. This is the case of Escherichia coli. Okamoto et al. (1965) working with supernatants of resistant strains of E. coli to chloramphenicol and dihydrostreptomycin, found inactivating enzymes against kanamycin. Also, it was observed that the inactivation of the drugs required Acetyl-CoA, suggesting an acetylation of some groups in the drug molecule.

Another antibiotic of the same group that was tested in cell free extracts of E. coli was gentamicin. As kanamycin, this antibiotic strongly stimulated the incorporation of amino acids in the presence of endogenous messengers and the stimulation of wrong amino acids incorporated with synthetic polyribonucleotides.

Based on the results from the resistant strains of

E. coli to gentamycin, it was suggested by Milanesi and Ciferri (1966) that this resistance is due to a mutation that affected a non-ribosomal protein. This idea was suggested since the interference seemed to take place in the interaction between the amino acid polymerizing enzymes and the RNA ribosome complex.

Most of these types of antibiotics are active against many gram positive and gram negative microorganisms including *Staphylococcus*, *Klebsiella*, *Aerobacter*, *Shigella*, *Salmonella*, *Nisseria*, *Escherichia*, *Proteus* and *Mycobacterium* (Takashi, 1977).

Cell Division

As cells grow they divide periodically. During the logarithmic phase of growth, the cell doubles its original mass and then separates into two. The average cell size can change sometimes due to environmental conditions or applications of drugs. These factors can advance or delay growth and in extreme cases, they can inhibit growth completely. The cell possesses an internal organization that is imposed by strong binding and orienting forces determined by a condition of minimum potential energy

(Sargent, 1978). In general, a great deal is known about the chemical components of the surface of the cell. However, the information available concerning control of surface area is in most cases, inconclusive.

Sargent (1978) stated five important factors concerning the growth and form of rod-shaped bacteria. They are as follows:

1. During growth in a steady state, length extension is continuous without change in width.
2. The timing of division is relatively casual with quite a large variation in age of individual organisms at division.
3. The variation in size at division is significant, but less so than age at division.
4. Division septa are formed almost exactly at the centre of a cell.
5. Average cell length and width vary with growth conditions.

Separation of Division and Growth

In some circumstances, the cell continues to divide after the exhaustion of nutrients and becomes smaller without an increase in total mass. This process is due to an imbalance between the ratio of DNA/mass and the ratio of

DNA/cell, the former rises and the latter falls. However, growth without division is the result of elongation, which in extreme cases can produce a bacterial cell of several hundred times the length of a normal cell. This condition of elongation can be due to drugs or the transfer of cells to a unaccustomed medium (Dean, 1966).

Factors Determining Average Cell Size

Previc (1970) cited in Sargent (1978), gave the first explicit suggestion regarding the significance of the increased mass per unit length at high growth rates in enteric bacteria. It was proposed that the growth zones, which are found at potential division sites, complete a unit cell length at the termination of chromosome replication. At this point, the surface extension at this site ceases while simultaneously two new growth zones are formed at the next potential division site. The division is associated with chromosomal termini. Based on this idea, many researchers have tried to visualize the critical connection between replication of the chromosomal termini and surface growth. For example, Zaritsky and Pritchard (1973) cited in Sargent (1978) suggested that the sites of length extension could operate in two possible ways:

1. At a constant rate proportional to the mass growth rate of culture such that $\bar{L} = K2^{D/T}$, (where: $\bar{L}$ = average cell length, t = generation time, K = constant and D = time between termination and cell separation), the circumference of the cell varied to accommodate the mass per unit length at the same density.
2. The rate of surface area would increase proportional to the output of an unregulated gene located in the middle of the chromosome.

Other theories have been proposed by the same authors, as described by Sargent (1978). The theories were presented as equations to explain the relationship between cellular dimension by parameters such as average length per cell, generation time, average surface area, chromosome replication time, time between termination and cell separation and a control point "X" which indicated the minutes before separation.

According to Sargent (1978) the relationship between growth rate and cell length can be fitted to straight line plots. However, it is suggested that the variations from a perfect fit are probably due to growth medium variations in the value of X. For example, in Bacillus subtilis, the

variation in X is considered to be the time between nuclear division and cell separation. That is, the X period starts at nuclear division, which probably coincides with chromosome termination. For E. coli, the X period probably starts at or close to chromosome termination.

There have been many investigations to clarify the relationship between dimension control and the chromosome cycle. Some experiments have been done with thymine requiring strains of E. coli (Sargent, 1978). Low concentrations of thymine decreased the velocity of chromosome replication without affecting the mass growth rate. Also, there is an increase in the interval between chromosome initiation and cell separation and a decrease in the interval between termination and cell separation. These changes accelerated septum formation.

As stated before, the cell size not only varied when an inhibition of protein synthesis occurred, but also when media composition and temperature varied and when the application of drugs occurred. For example, nitrogen-limited enteric bacteria can have a greater mass per cell than carbon-limited cells at the same growth rate (Sargent, 1978). In media with phosphate and tryptophan limitations,

bacteria can reach a maximum size at a growth rate of about 0.6 generation per hour. In the literature, reviewed by the same author, it is stated that there is a tendency for several organisms to form filaments at the high and low extremes of their temperature range.

In gram negative bacteria, a number of related antibiotics produce different morphological effects at their lowest inhibitory concentration. For example, cephalonthin and cephaloridine can cause severe lysis. That is, specific inhibitors of peptidoglycan synthesis can cause filaments in E. coli, Salmonella typhimurium and Clostridium perfringens, as well as swellings of Streptococcus and Staphylococcus species with inhibition of cell separation. Another example of these changes is the case of β-lactam antibiotics that have been divided on the basis of their morphological effect and their affinity for three of the penicillin-binding proteins found in the E. coli membrane. Other compounds that produce long-cell filaments are proflavine, m-cresol and tertiary butyl alcohol (Dean, 1966). It is evident that a variety of factors play an important role in the changes of length, width and mass of a cell.

Sargent (1975) established that the length of a rod-shape organism is determined by the following factors:

a) the number of growth zones

b) the rate of length extension per growth zone

c) the time in each cycle over which length extension occurs.

Thus, an increase in mass, length and defective septum formation will be the result of an increase in the time between chromosome termination and cell separation, while an increase in the chromosomal replication time is the result of an increase of cell width (Sargent, 1978).

MATERIAL AND METHODS

1. Culture

The main working culture of Erwinia carotovora was kindly donated by Dr. Roma Hawirko of the Microbiology Department, University of Manitoba. Bacterial cultures were maintained on nutrient agar slants and stored at 4°C. New working slants were inoculated with Erwinia carotovora every 7 - 8 weeks. Before every test, the microorganism was grown in nutrient broth for 24 hours and before inoculation a gram stain was done. Maltotriose, purchased from Sigma Chemicals, St. Louis, Mo., was autoclaved at 121°C for 15 minutes, no stability problems were encountered using this method of sterilization.

The wave length of maximum absorption was determined by scanning a 24 hour E. carotovora culture, from 325 to 700 mm, using an UNICAM SP 800 Ultraviolet double beam spectrophotometer. The culture was run against an uninoculated medium reference. Based on the results obtained, the wave length chosen for all further work was 373 nm.

Reducing sugars, gram stain, nitrate reduction, motility and gas production tests were done following the methods from the Difco Manual (Anon., 1953). Test for

differentiation of Erwinia carotovora was performed as described by Burkholder and Smith (1949). The medium used consisted of: beef extract - 3g, peptone - 5g, bromothymol blue - 0.01%, ethanol - 5% and 1000 ml of H_2O. The medium was sterilized at 121°C for 15 minutes. The test was done in triplicate by adding 200 ml of medium to 500 ml flasks. Each flask was inoculated with a 0.5 ml of a 24 h culture that contained 10^6 cells/ml. The flasks were put in an incubator, on a shaker at 100 rev/min., at 27°C for 7 days.

The rotting ability of the working culture was tested in triplicate on potato and cucumber slices. The knife used for both vegetables was dipped in alcohol and then flamed, potatoes were likewise flamed before slicing but cucumbers were not. The slices were permitted to fall into a sterile petri plate and then immediately inoculated with a 24 h culture and incubated at 27°C for 48 hours. Pathogenicity tests were performed with potato slices every time new slants were prepared. Tests on cucumber were done at the beginning of experimental work.

2. Media

A minimal medium was employed consisting of glucose 3.5% w/v, NH_4NO_3 0.7%, KH_2PO_4 0.4% and $MgSO_4$ 0.2% as a base, several tests were done to obtain an otpimun medium for growth and for turbidity readings. Repetitive turbidity readings were difficult to obtain because at certain unknown conditions, the medium suffered a slight color change, giving variable results.

The nitrogen source was changed from an inorganic to organic form. The compounds tested at different concentrations were:

NH_4NO_3 - 0.7% w/v, 0.9%, 1.1%

Proteose peptone No. 3 - 0.7% w/v, 0.8%, 0.9%

Peptonized milk - 0.05% w/v, 0.1%, 0.2%, 0.4% 0.5%

The carbon source (glucose) was also tested at the following concentrations - 1.5% w/v, 2.5%, 3.5%, 4.0% and 5.5%. With the purpose of adaptation of the microorganism, the 24 h culture used in each experiment was prepared in the same medium as the one used for the test.

Six different media, with and without 3G (5000 μgm/ml) were inoculated to follow pH changes. The media used were nutrient broth, trypticase soya broth, nutrient broth with

glucose 3%, potato medium and cucumber medium. The last two media were prepared as follows: two potatoes (150 - 200g) were washed in tap water, cut in fourths and placed in a blender. Then 50 ml of tap water was added and the mixture was blended for 2 minutes. The mixture was then filtered through cheese cloth in a Buchner funnel until 200 ml of the filtrate was collected. The cucumber medium was prepared exactly in the same way, but no water was added to the blender. All six media were autoclaved at 121°C for 15 minutes, cooled and inoculated with 1 ml each of a 24 h culture, grown in nutrient broth.

Shake cultures on nutrient broth with 3G were tested for residual inhibitor after 24 h of growth. Thin layer chromatography (TLC) was employed using cellulose plates (Eastman Kodak #6064) with a solvent of ethyl acetate: propanol: water at a ratio of 1:6:3. The spray reagent used to detect 3G was aniline-diphenylamine - phosphoric acid. Attempts to estimate the amount of 3G on the TLC plates were qualitative only.

3. Inhibition of Erwinia carotovora in Solid Phase

For all in-vitro tests performed, the following minimum media (MM) was used: glucose 3.5%, peptonized milk 0.4%,

KH_2PO_4 0.4% $MgSO_4$ 0.2% and agar 1.5%. Except for glucose, all the components were sterilized at 121°C for 15 minutes. Glucose was sterilized by a Millipore filter (0.45 nm) and then added aseptically to the medium. The agar was poured into petri plates and these were inoculated with 1 ml or 0.5 ml of a culture that contained 10^5 cells/ml, prepared in the same type of medium. The culture was distributed with a sterile glass hockey stick. The 3G was used in a concentration of 5000 μgm/ml and was applied in two different ways:

		3G added (ml)		
a)	On sterile disks (Bacto concentration disks 1/2")	0.1	0.15	0.2
b)	In wells	0.1	0.2	0.4

The disks were placed on to the agar immediately after the plate was inoculated or after the inoculum had dried at room temperature for 5 minutes. 3G was applied with a 2.2 ml pipette on to the disks. In the case of wells, sterile cork borers of different sizes were tested until the volume of 3G was at the same level as the surface of the agar. Two sets of plates were prepared and incubated for 48 hours, one set was held at 10°C while the other was at 27°C.

For photography purposes, the same agar was supplemented

with bromothymol blue or phenol red at 0.1% and 0.05% with the following indicator ranges:

	pH	color
bromothymol blue	6.0	yellow
	7.6	blue
phenol red	6.8	yellow
	8.4	red

During preliminary testing, the results of these plates were not satisfactory due to rapid diffusion; therefore, the following substances were added to the minimum agar in an attempt to suppress diffusion:

Minimum agar + 2.5% agar

Minimum agar + 2.5% NaCl

Minimum agar + 2.5% glycerol

Minimum agar + 3% glycerol + 2.5% NaCl

Minimum agar + 3.5% NaCl

Plates were incubated at 27°C for 48 hours. Results of this phase of the study were not satisfactory for photographic purposes. For this reason, 1% starch was added to the minimal medium. These plates were treated exactly as before, except for the size of inoculum, which in this case, was 10^5, 10^6, 10^7 and 10^8 cells/ml, with 0.5 ml of each

concentration. Maltotriose solution (0.1 ml) at 10,000 μgm/ml and 5000 μgm/ml were added to the disks that were put on the dried agar plates. The plates were incubated at 27°C for 48 hours. After incubation, the plates were treated with 5 - 7 ml of 0.5% Iodine for 5 seconds and then washed.

Potato dextrose agar, was also used to demonstrate inhibition. Plates and inoculum were prepared as described before. The inoculum contained 10^5 cells/ml and after the maltotriose was added to the disks, the plates were incubated at 27°C and 10°C for 48 hours.

Finally, the effect of maltotriose was tested on potato slices. To obtain a horizontal surface, the tuber was sliced with a Hobart meat slicer. The potato was immersed in ethanol, flamed and sliced, while the slicer was cleaned with 95% ethanol just before it was used. The sterile slices were inoculated with the test organism. Then the antibiotic, 0.15 ml of a 10,000 μgm/ml solution was applied with a 2.2 ml pipette by two methods: immediately after the disks were put on to the inoculated potato and before the disks were put on the potato. The plates were incubated at 27°C for 30 hours. All controls were prepared exactly the

same way, but water was used instead of 3G.

4. Cellular Morphology

Morphology was followed during growth under various conditions. Cell changes were monitored using light microscopy (Reichert, Austria Nr. 54581 Nr. 321961 TLI 10X - 5.0/0.40 MM, TLI 40X - 5.0/0.12 MM) and electron microscopy (AEI-6B). In both cases, photomicrographs were made. For light microscopy, the cells were routinely stained using the Gram method, while for electron microscopy, the cells were treated with 0.1 M cacodylate buffer ($Na(CH_3)_2\ AsO_2\ 3H_2O$), 2% noble agar, 1% osmium, then they were embedded in spur plastic and sectioned. Cellular counts were done using Petroff-Hausser and Helker counting chambers. Other indicators of growth (gram stain, pH, absorbance at 373 nm) were routinely done during these cell morphology studies.

For comparative purposes, controls, i.e., growth samples without 3G were maintained in order to have cells from non-inhibited systems. The samples were prepared in 500 ml flasks as follows: 75.8 ml of MM + 11.6 ml of glucose (30g/100 ml) + 12.5 ml of 3G (2g/50 ml). The control was

prepared using 88.4 ml of MM and 11.6 ml of glucose solution. The flasks were inoculated with 10^5 cells/ml and incubated at 4^oC, 10^oC, 27^oC and room temperature. For each temperature, two sets of samples were performed. One sample was put on a shaker at 100 rev/min while the second sample was not shaken.

5. Minimum Inhibitory Concentration (MIC)

A modified MIC test was done following the Antibiotic-Susceptibility Testing method of Faulkner and King (1970). The samples were prepared as follows:

Tube No.	Minimal[a] Medium (ml)	Inoculum (ml)	3G[b] (ml)	H_2O (ml)	Final 3G Conc.
1	4.5	0.5	5.0	0	10000
2	4.5	0.5	4.0	1.0	8000
3	4.5	0.5	3.0	2.0	6000
4	4.5	0.5	2.5	2.5	5000
5	4.5	0.5	2.0	3.0	4000
6	4.5	0.5	1.0	4.0	2000
7	4.5	0.5	0.5	4.5	1000
8	4.5	0.5	0.25	4.75	500
9	4.5	0.5	0.125	4.875	250
10	4.5	0.5	0	5.0	0

[a] The minimal medium and the glucose were sterilized separately and mixed before they were poured into sterile tubes.

[b] Initial concentration was 10,000 gm/ml.

The MIC test was performed at 27°C, 10°C and 4°C.

RESULTS AND DISCUSSION

1. Biochemical Tests

The various biochemical tests to characterize the working strain of Erwinia were as follows:

Reducing sugar	+
Gram stain	-
Nitrate reduction	+
Motility	+
Gas production	-
Acid production	+
Rotting ability	+
Ethanol consumption	+

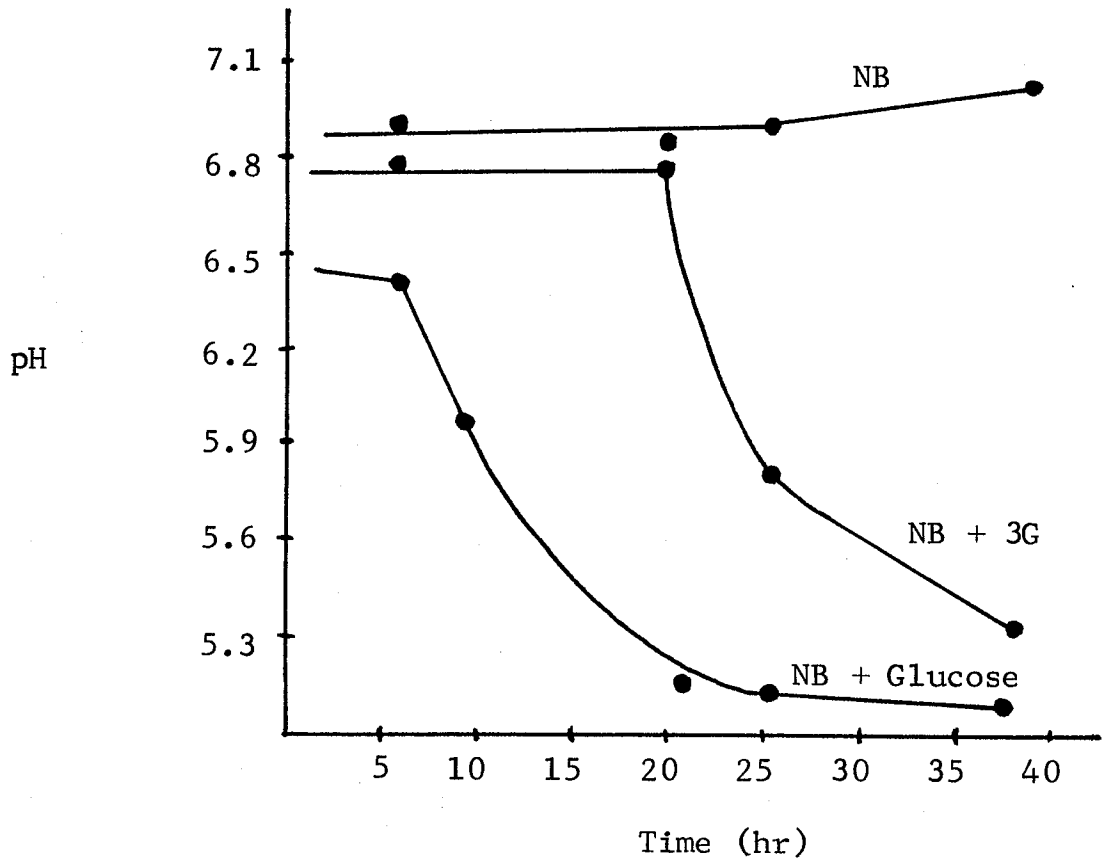
The chemical tests done on this microorganism were satisfactory to demonstrate the species carotovora, however, they were inconclusive to show a difference between varieties. The positive test for reducing sugar indicated the variety atroseptica, while the consumption of ethanol suggested the variety carotovora. Such biochemical varieties seem to be a characteristic of the genus Erwinia and both biochemical work done here and different literature references clearly indicate complex

and variable physiology chemistry for members of this genus. Although the organism used throughout this study was certainly Erwinia carotovora the actual variety is not clear. Considering the nature of this investigation, it was felt that this uncertainty was not a significant factor.

2. Growth Conditions

When the test organism was grown in a liquid medium, the pH changed always to the acidic side, except for nutrient broth where the pH remained always neutral. When inhibitor was added to the different media, an acid pH was also observed. Higher acidic values were obtained when the inhibitor was added than when it was not present. This finding suggests a breakdown of maltotriose and therefore, the production of glucose and the end products of its fermentation. To verify this fact, glucose was added to nutrient broth instead of maltotriose. The same results were obtained suggesting that maltotriose and glucose were fermented and the end products resulted in the acid pH (Fig. 2). However, when a qualitative thin layer chromotagraph was done with a zero hour and a 17 hour

Figure 2. Changes in pH when Erwinia carotovora was Grown in Nutrient Broth Alone (NB) and Supplemented with Either Glucose or Maltotriose (3G)



culture, it was found that maltotriose was still present in both samples. Indeed, the size of the 3G spot did not seem to decrease with growth.

It would appear that the fermentation of maltotriose was not taking place although a partial inhibition did occur as seen in Fig. 3 suggesting that this type of inhibition was a more complicated one than an inhibition due simply to acid production, as first speculated. When 3G was added to both potato medium and a cucumber medium, similar results were obtained but the difference in pH values between samples with and without 3G was not significant. This fact is probably due to the low amount of nitrogen compounds in the vegetables (Fig. 4).

In all solid media, except in tests where glycerol was added to the agar to prevent diffusion of brom thymol blue, the pH values for solid media tested (including potato slices) were alkaline. In the case of potato slices, this change of pH contributed to the development of soft rot, because the optimum pH for the activity of pectinases is on the alkaline side (Gregg, 1952). In tests where glycerol was added to solid media, the resulting acid pH would indicate the use of glycerol by Erwinia and the

Figure 3. Changes in Absorbance When Erwinia carotovora was Grown in Broth Alone (NB) and with Maltotriose (3G)

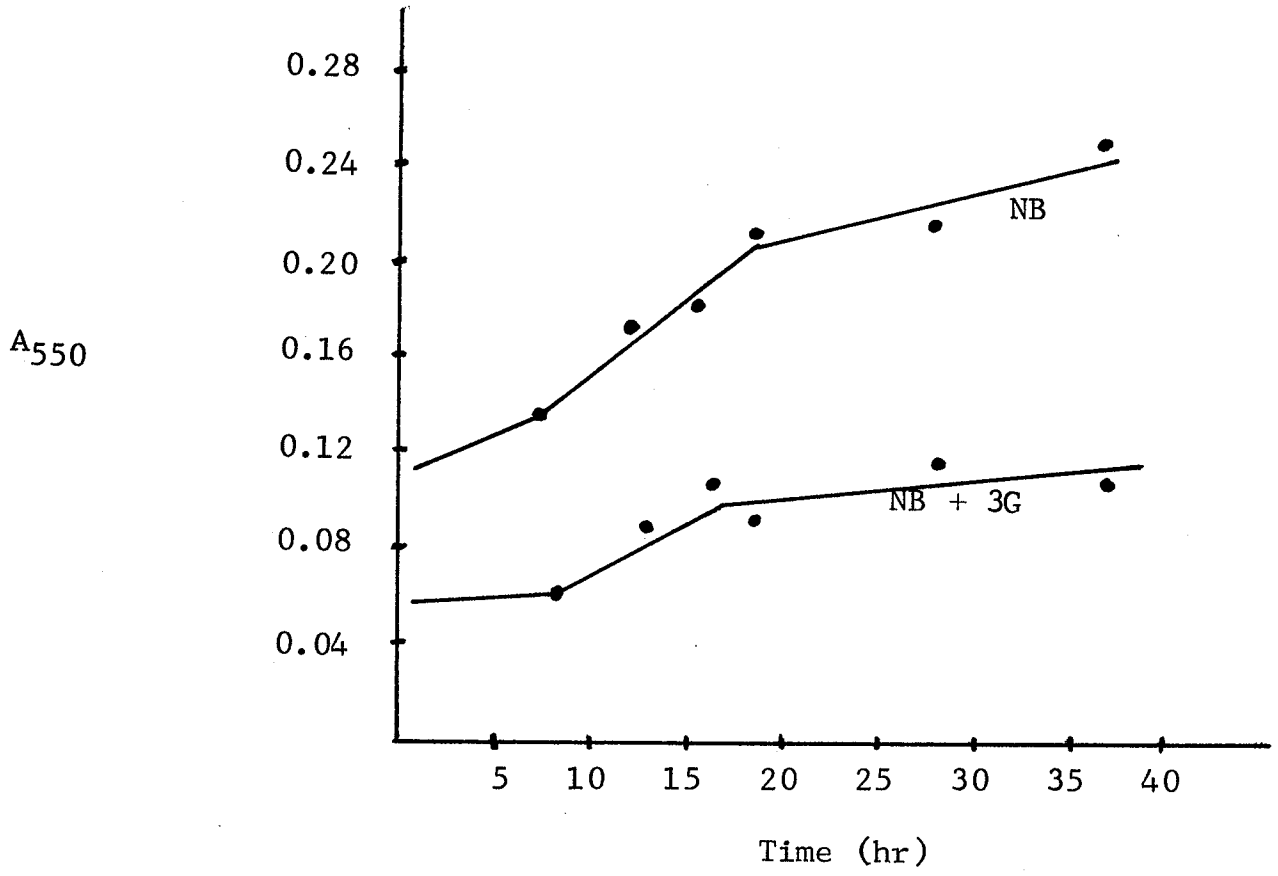
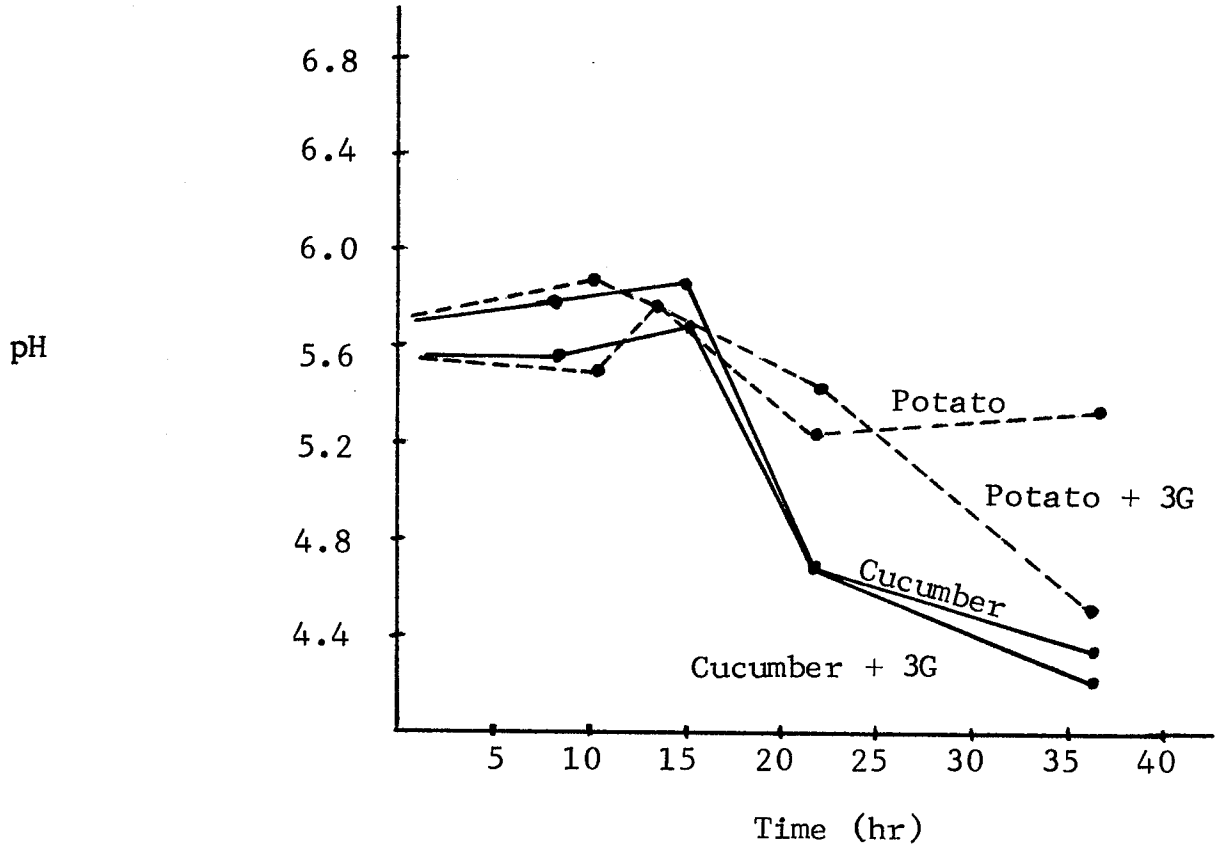


Figure 4. Changes in pH when Erwinia carotovora was Grown in Cucumber Medium and Potato Medium, Both Media Were Used Alone or with Maltotriose (3G)



production of acidic products. This result was opposite when compared with the one obtained by Lelliot (1974), indicating again variation of biochemical tests.

When several concentrations of glucose plus inorganic and organic nitrogen sources were tested in minimal media, it was found that the best medium for a short lag period and practical turbidity readings consisted of:

glucose	3.5% w/v
peptonized milk	0.4%
NaH_2PO4	0.4%
Na_2SO_4	0.2%

In general, higher concentrations of glucose lead to a high acid pH, while a high concentration of the nitrogen source leads to an alkaline pH value. Both extremes of pH resulted in a loss of motility and in very small cells of Erwinia carotovora. Low concentration of both compounds resulted in a long lag period, which clearly indicates an imbalanced medium. The effect of different concentrations of the other two components of the media (NaH_2PO4 and Na_2SO_4) were not tested.

In other complex media tested, turbidity readings were inconclusive since there was a slight change of color of

the media and sometimes the uninoculated medium had a darker color than the inoculated one. However, the sterile minimal medium had only a light yellow color due to the peptonized milk and was therefore, considered to be a good medium for turbidity readings. In order to obtain better results in this type of experiment, it is suggested that one blank with 3G and one blank without 3G be used, this corrects for 3G dilution effects.

3. Inhibition in Solid Media

Total inhibition, as shown by the formation of inhibition zones, occurred in all solid media tested except for the PDA medium (Table 3). Inhibition tests were done using either wells in the agar or filter paper disks. The results obtained using the well method were not satisfactory. It was observed that the solution of 3G poured into the well was absorbed all along the well and into the agar, no inhibition occurred. It is suggested that the lack of inhibition was due to an insufficient amount of 3G in the zone around the well, due to a rapid diffusion across the agar.

Inhibition zones were observed around the paper disks.

Table 3

Relationship Between Concentration of 3G and Cell Number in vitro

Cells/ml	Concentration of Inhibitor (μgm/ml) 2500	5000	10000
10^8	-	-	+
10^7	++	++	+++
10^6	++	++	+++*

\- No inhibition

\+ Small halo of inhibition (1 mm)

++ Regular halo of inhibition (1 - 2 mm)

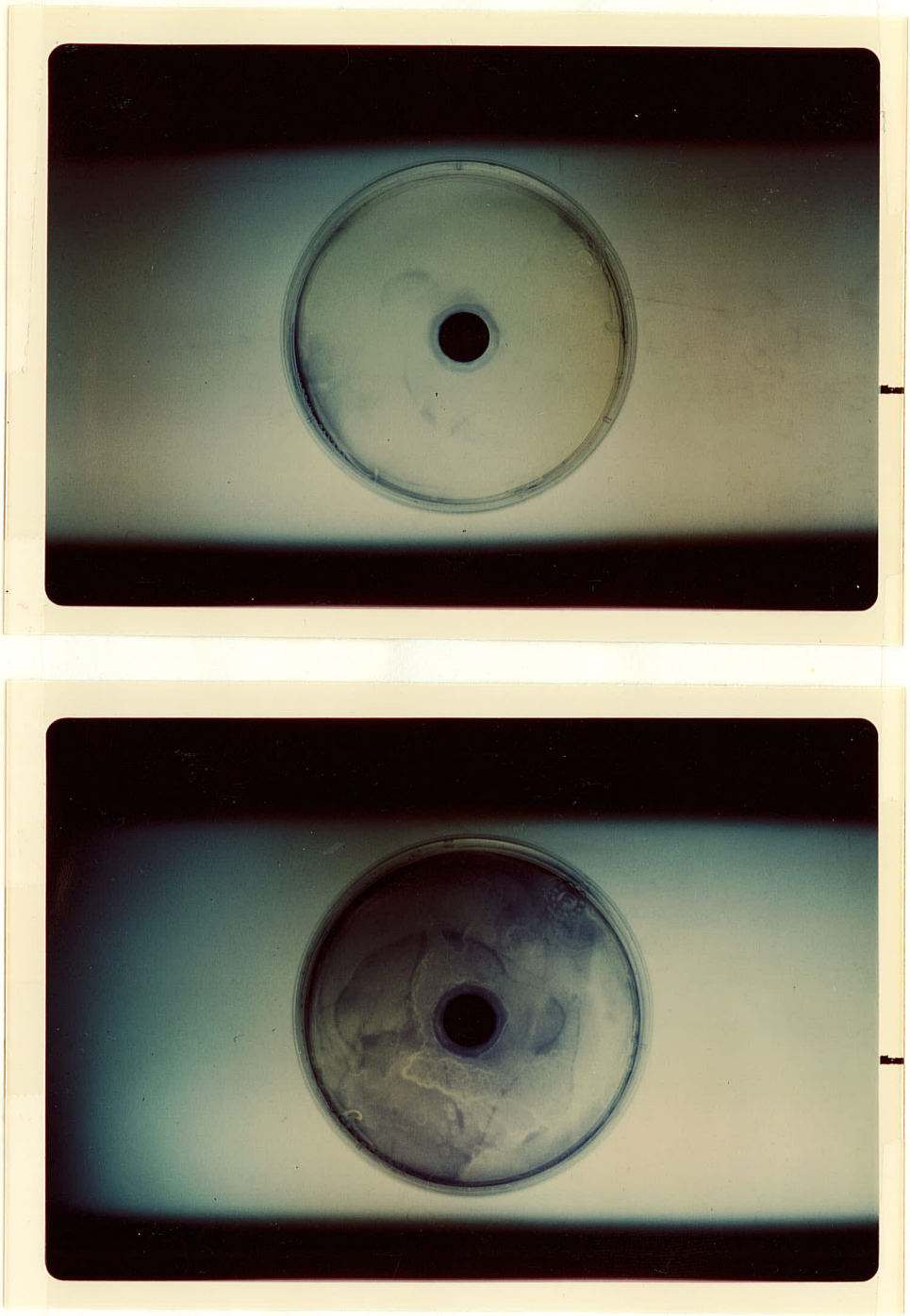
+++ Big halo of inhibition (2 - 3 mm)

+++* Double size (4 mm)

Many attempts had to be made in order to establish very clear zones. The best results were obtained when two drops of a stock solution containing 5000 μgm/ml of 3G were added (i.e. 500 μgm of 3G) to the disk with a sterile pipette. With two drops, two conditions were achieved: 1) a sufficient amount of the inhibitor was present to produce the desired effect; 2) the formation of a meniscus between the disk and the agar was avoided. It was observed that when this meniscus was formed, the solution did not spread on the agar and therefore, no inhibition occurred.

The addition of the two indicators, phenol red and brom thymol blue, was done in order to observe color differences between the zone of inhibition and the zone of growth. The results obtained were not satisfactory due to the presence of alkaline end products which diffused rapidly into the entire agar plate. For this reason, the zone of inhibition was not clear. To avoid the total change of color, due to the presence of alkaline products, different compounds were added to decrease agar migration of metabolites. The clearest zones were observed when 2.5% w/v NaCl was added, but the results were still not

Figure 5. In-vitro Inhibitory Effect of 500μgm of 3G Minimal Medium Enriched with Starch



clear enough for definite experimental purposes. Therefore, the use of pH indicators to bring out inhibitory zones, was abandoned.

Another approach attempted was to add 1% w/v potato starch to the minimal medium. When an iodine solution was poured into the petri plate and then washed off after 10 seconds, it was observed that in the zone of inhibition there was no starch breakdown and, therefore, the zone remained dark while the rest of the petri plate turned white-yellowish in color (Fig. 5).

In the case of a more complex medium (PDA) inhibition zones were not observed, because there was always a cellular overgrowth, suggesting that this is a very nutritious medium for Erwinia carotovora. In tests done using whole potato slices as a growth surface, zones of inhibition were observed when 3 drops of 10,000 μgm/ml solution of 3G (i.e. about 1500 gms total) were added to each disk.

The amount of moisture on the surface of the potato slice was found to be very important for cell growth. For this reason, it was necessary to apply the solution of 3G onto the disk, before it was placed on the potato slice.

Otherwise, the disk will absorb the inoculum that contains a high moisture, and the resulting zone of inhibition would be very small. The potato slices had inhibition zones of 2-3 mm in diameter.

The rot produced on the slices presented a black ring around the white zone of cell growth and tissue breakdown, as described by Lovrekovich et al. (1967). Around the outside of the black ring the starch granules could be easily observed in a stereoviewer which indicated the absence of surface microorganisms. This confirmed the results of Lovrekovich et al. (1967), who suggested that the ring was involved in the defense mechanism of the vegetable. Starch granules were also observed readily around the disk that contained 3G, indicating the inhibition in-vivo (Fig. 6).

4. Morphological Changes

The cells grown in liquid minimal medium in the presence of 5000 μgm/ml of 3G underwent a morphological change that was expressed as an elongated cell. The cells started to enlarge approximately 17 hours after inoculation and the long forms started lysing 8 to 10 hours later (Fig. 7). The actual cell length varied from two times to

Figure 6. In-vivo Inhibitory Effect of 1500 μgm of 3G on Minimal Medium Enriched with Starch

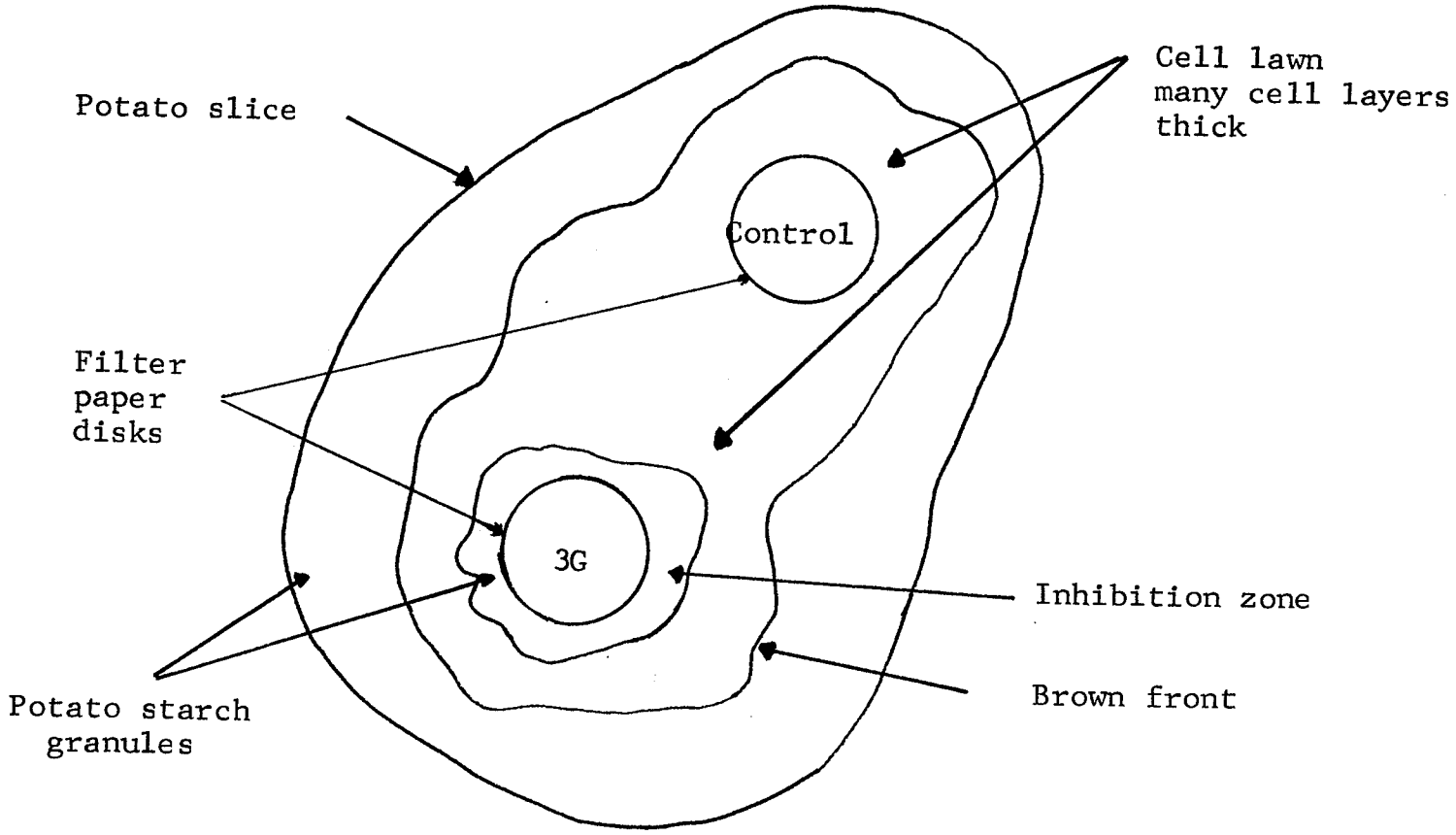
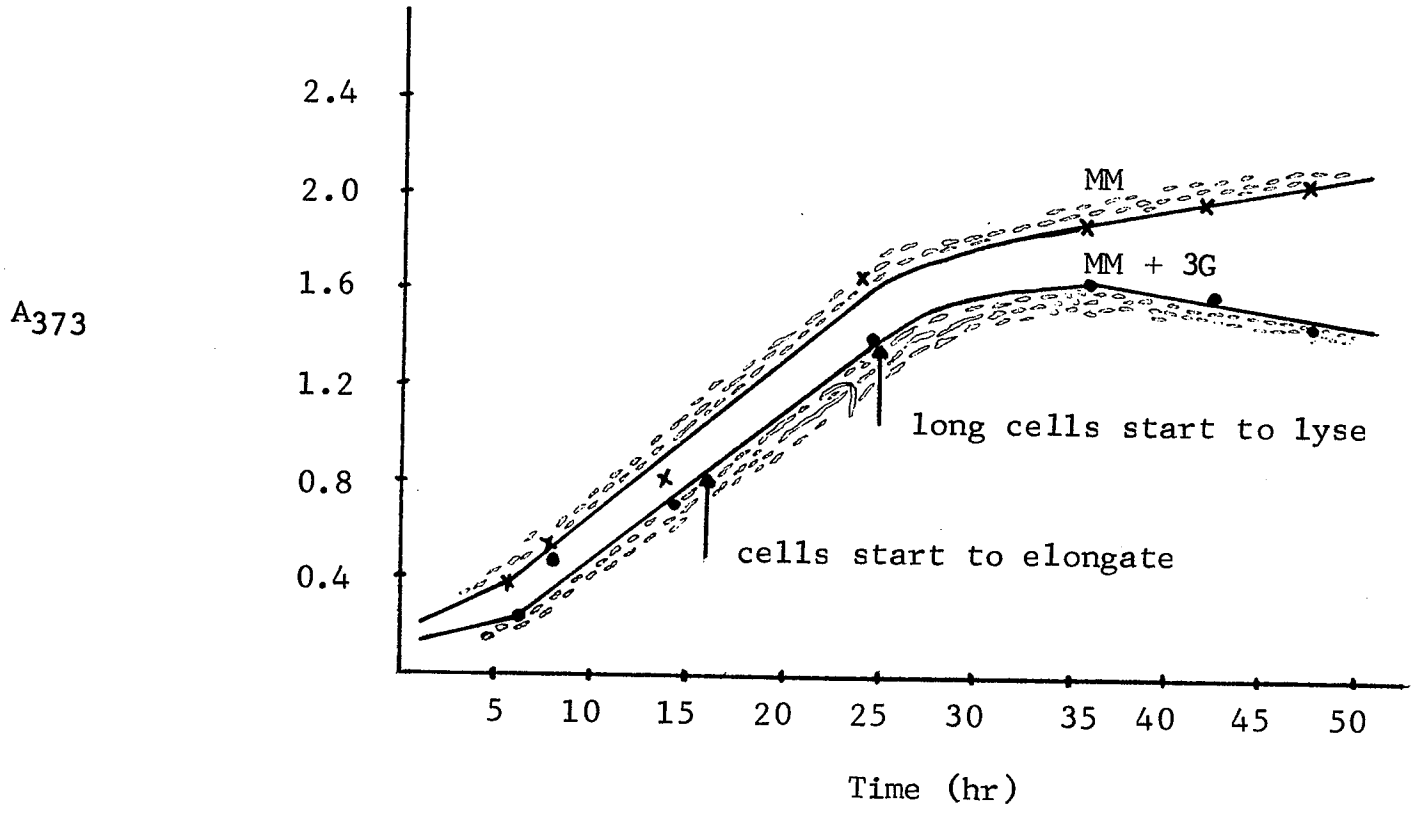


Figure 7. Absorbance and Morphological Changes During Growth of Erwinia carotovora with Control and Inhibitory Conditions



several times the original size, while the width remained constant. After the cells lysed the remaining cells were either of the original uninhibited size or else much smaller as viewed with the light microscope.

The cell count and the absorbance of these growing cultures (Figs. 8 and 9) reflected a partial inhibition of E. carotovora, when compared with the samples that had no inhibitor. From Appendix 1, it can be seen that the absorbance values decreased after the long cells lysed. It would appear that this lysis is the cause of the decreased absorbance values. The fact that the values for MM with and without antibiotic are almost the same during the first 30 hours, suggests a higher absorbance in the medium with 3G due to the presence of long cells. In an inhibited growth system (Fig. 10a) during the early stages of the elongation phenomenon most of the cells achieved a longer size than that seen in controls (Fig. 10b), while in later stages, the number of long cells started to decrease and the number of smaller cells started to increase (Fig. 10c). However, during the final stages of growth, very long cells could still be observed (Fig. 10d) but after 36 hours, no long cells were found (Fig. 10e).

Figure 8. Direct Microscopic Counts During Growth of Erwinia carotovora with Control and Inhibitory Conditions

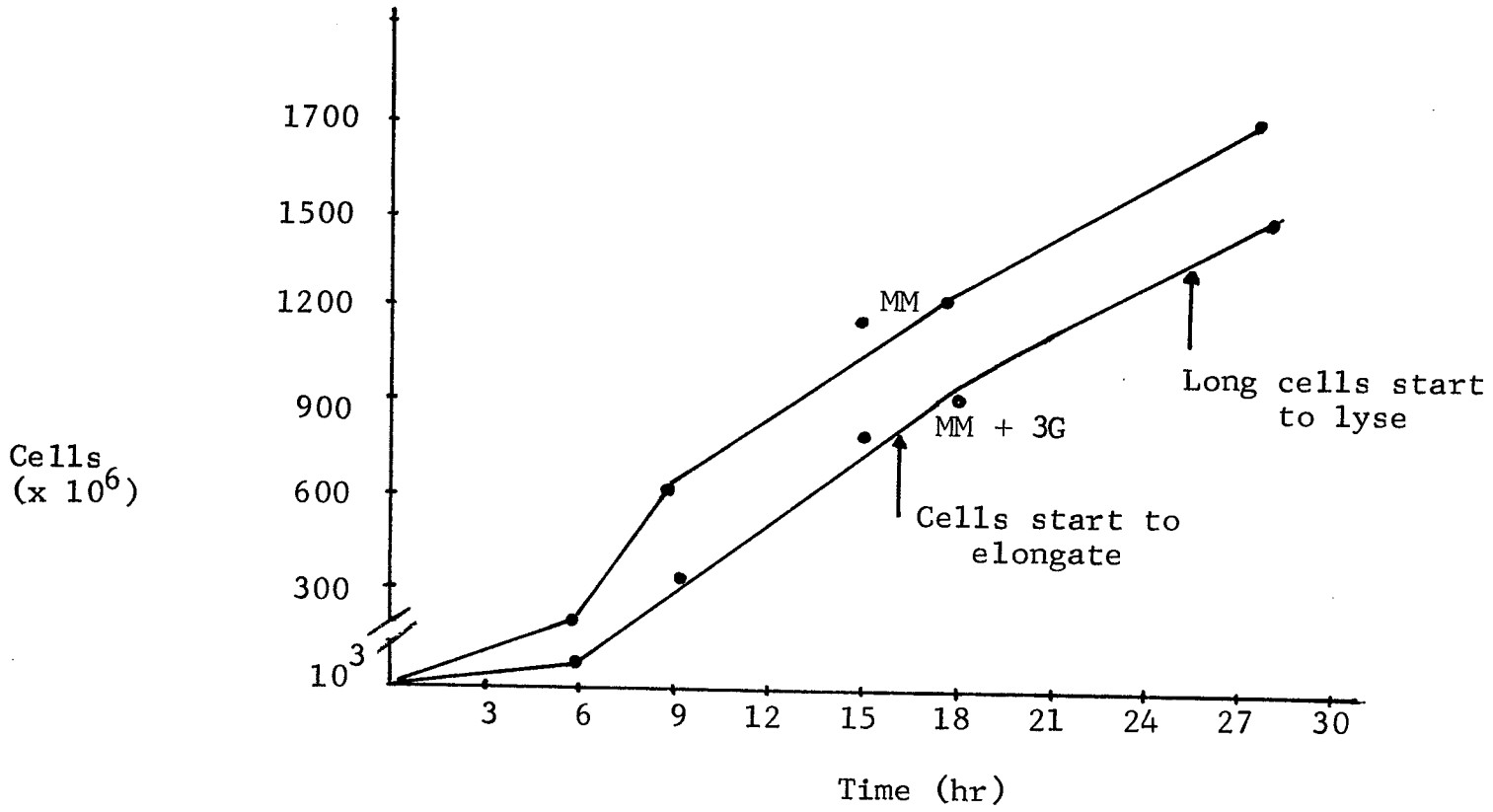


Figure 9. Absorbance Changes During an Extended Growth Period with Control and Inhibitory Conditions

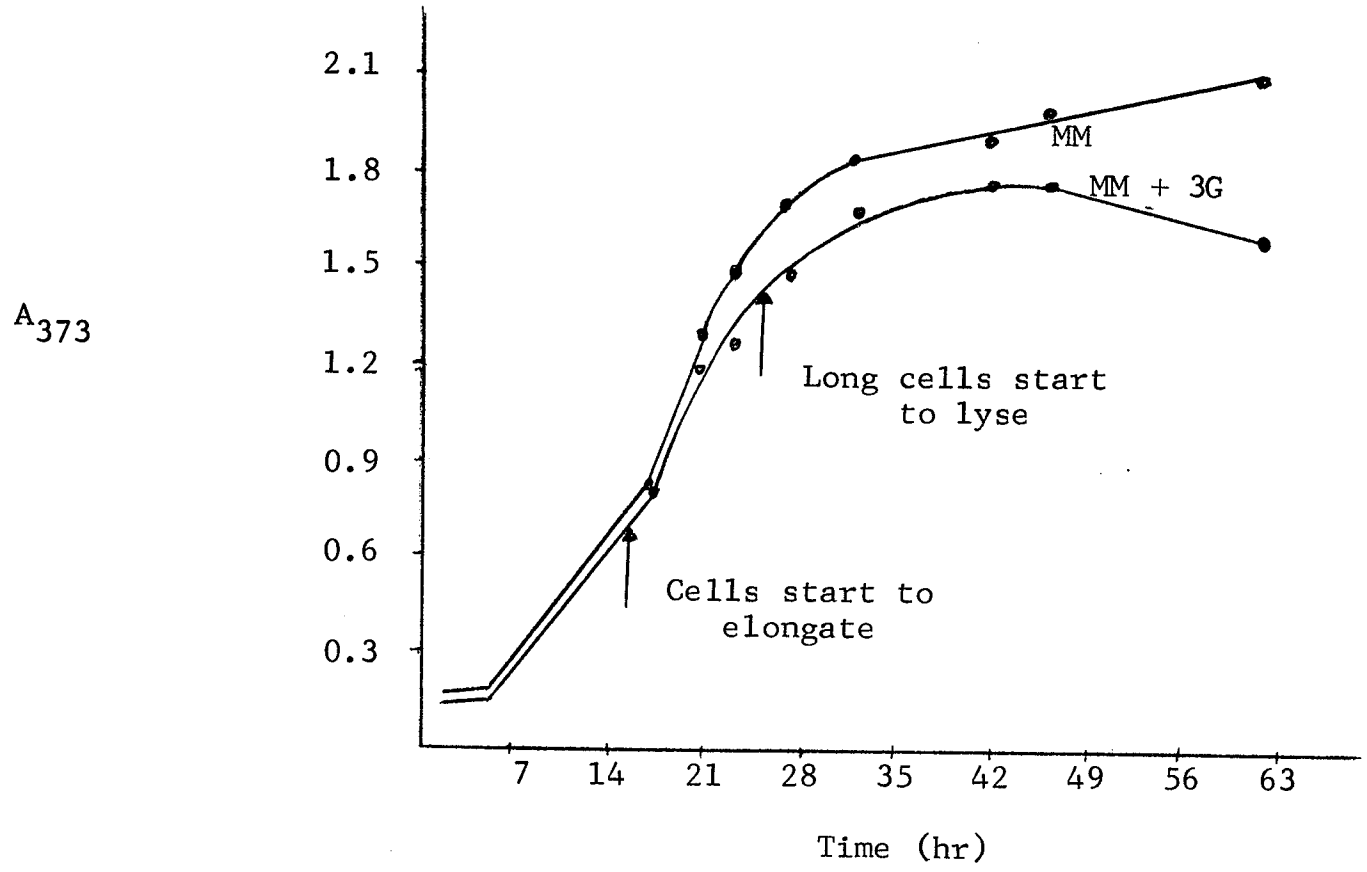


Figure 10. Photomicrographs of Erwinia carotovora all Magnifications (Microscopic and Photographic) are 630X.

a) Inhibited System at 17 Hours

b) Control System at 17 Hours

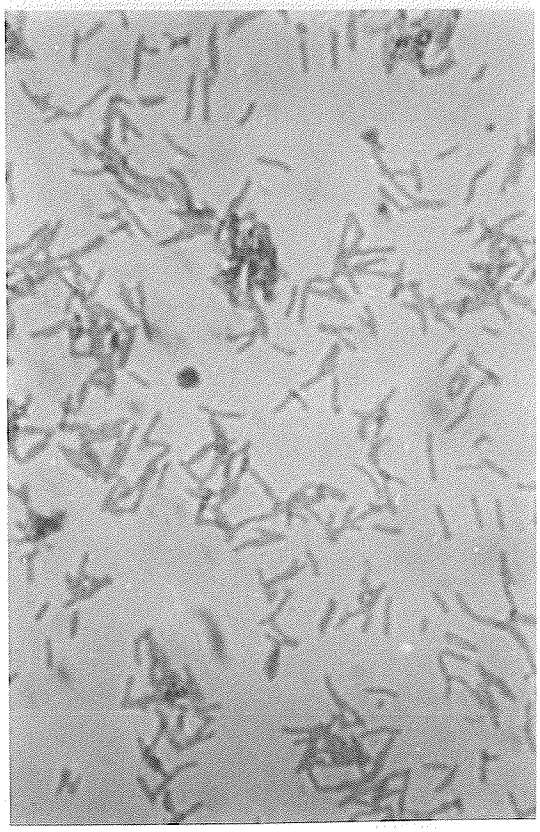
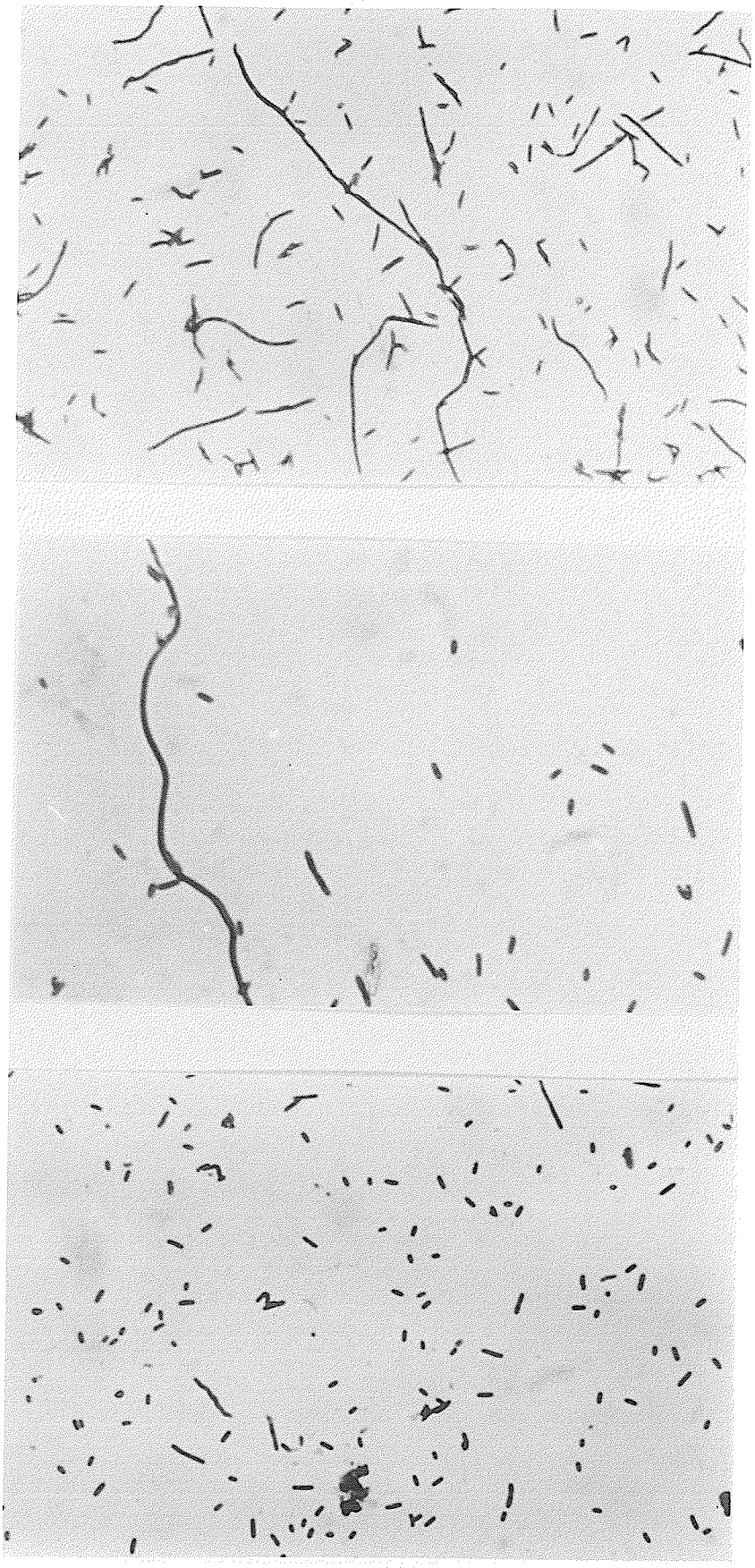


Figure 10 cont'd.

c) Inhibited System at 23 Hours

d) Inhibited System at 28 Hours

e) Inhibited System at 36 Hours

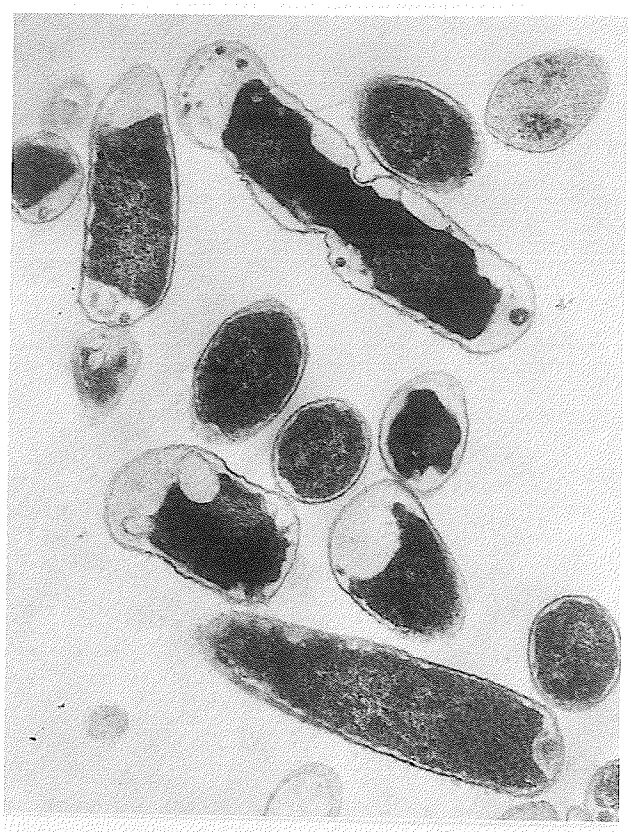


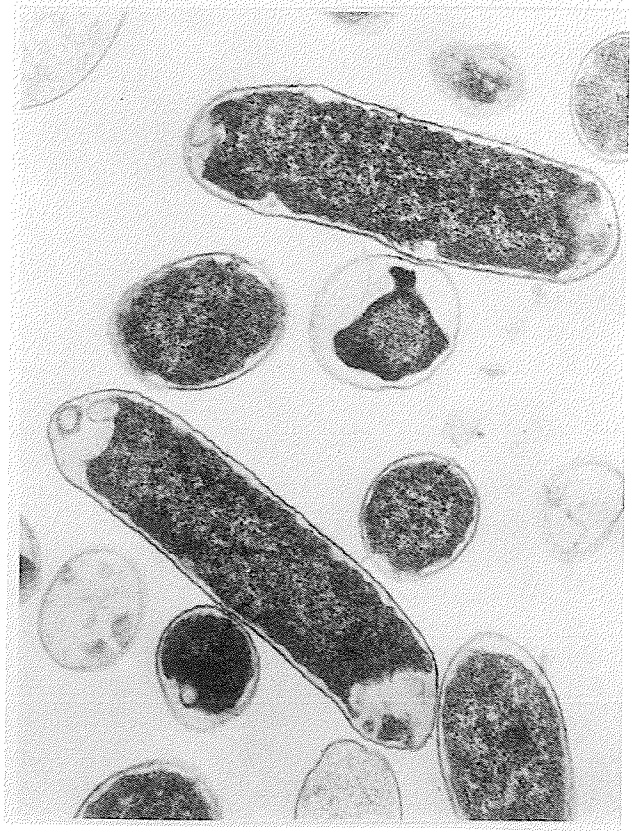
From transmission electron micrographs, it can be seen that the effect of 3G not only caused a change in length, but also a change in the internal structure of the cell. The cells that were grown in medium without 3G (Fig. 11a and b) presented a dark cytoplasm that covered almost all the cell except for the poles and the cytoplasm appeared to be separate from the cell membrane. This last point was also noted in the transversal sections. Spherical light bodies in most of the cells were also observed which could possibly be storage structures of fatty acids. When the cells from these electron micrographs are compared with other bacterial cells in general, it was observed that many cells grown in a medium lacking a specific metabolite presented certain similarities with the cells of Erwinia carotovora. It is then suggested that the minimal medium which was chosen for the purpose of turbidity readings was not the most optimum medium for the growth of Erwinia carotovora. However, it is also possible that the Erwinia cells grown in this study could be the normal cells of this microorganism as no comparisons between other electro-micrographs could be done, since those could not be found

Figure 11. Transmission Electron Micrographs of Erwinia carotovora, Total Magnification (Microscopic and Photographic) is 30,000X (a - b) Erwinia carotovora Grown in Minimal Medium

a)

b)





in the literature.

The cells that grew in the presence of 3G presented the typical cytoplasmic characteristics of other cells as observed in electromicrographs (Figs. 11c and d) except for the unusual length. The difference in both cases suggested not only a morphological change, but also a physiological one. The long cells shown here do not represent the total size as the ends of the cell were cut away during sectioning, making it impossible to see the complete length. The cytoplasm of these cells fills the entire cell volume and it is limited by the membrane.

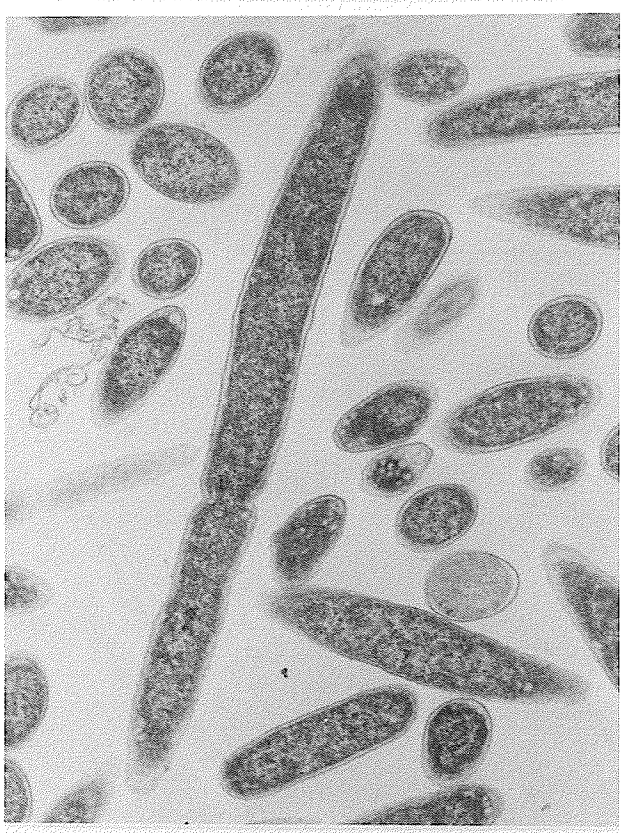
The elongation of cells is a phenomenon not completely understood, however, it is well known that certain antibiotics can cause this morphological change. Aminoglucoside antibiotics are known to cause an imbalance in the synthesis of protein due to a codon misreading (Tanaka, 1967 plus Milanesi and Chiferri, 1966). The similarity in chemical structure between the trisaccharide 3G and the aminoglucoside antibiotics suggests that a similar phenomenon may be occurring.

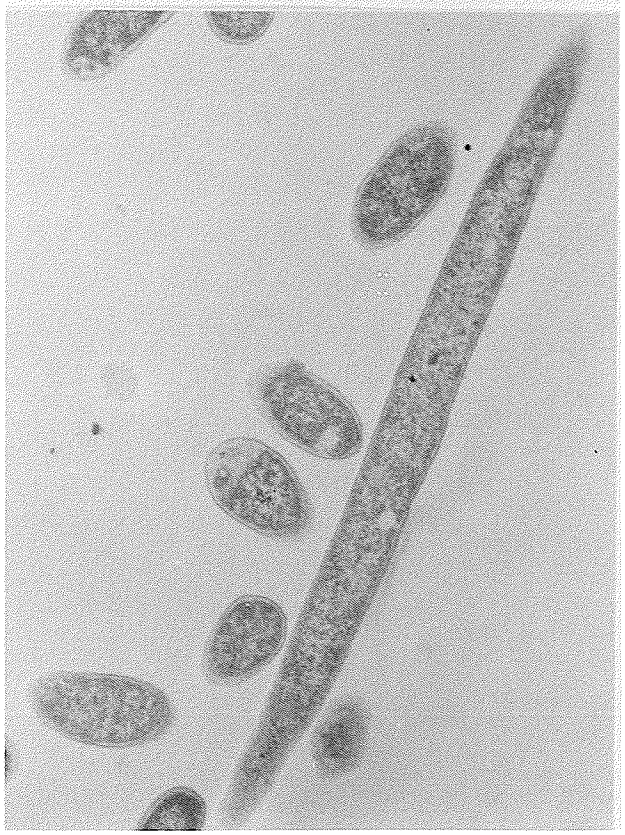
At present, it has not been possible to determine whether this elongation is related to a membrane over-

Figure 11 cont'd. (c - d) Erwinia carotovora Grown in Minimal Medium with Maltotriose (3G)

c) Note septum formation in central elongated cell.

d)





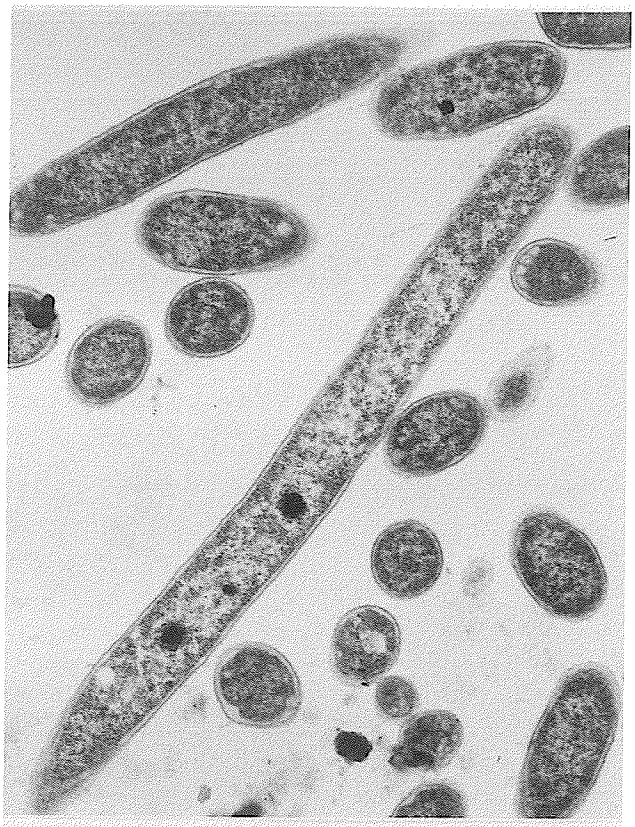
production, to cell division or to some other irregular metabolic process of the cell. It could be suggested that an overproduction of membrane occurred due to the strange morphological forms observed (Figs. 12 and 13). Extra membrane formation has been found in E. coli (Wergand et al., 1970) during a thermal mutation resulting in vesicular and whorl-like forms similar to the structures found in E. carotovora. The nature and function of extra-membrane, as well as its relationship with the division cycle, are still unknown. However, extra-membrane formation has been known to occur in cases of cell elongation and must be considered as a possible explanation for the phenomenon observed in this study.

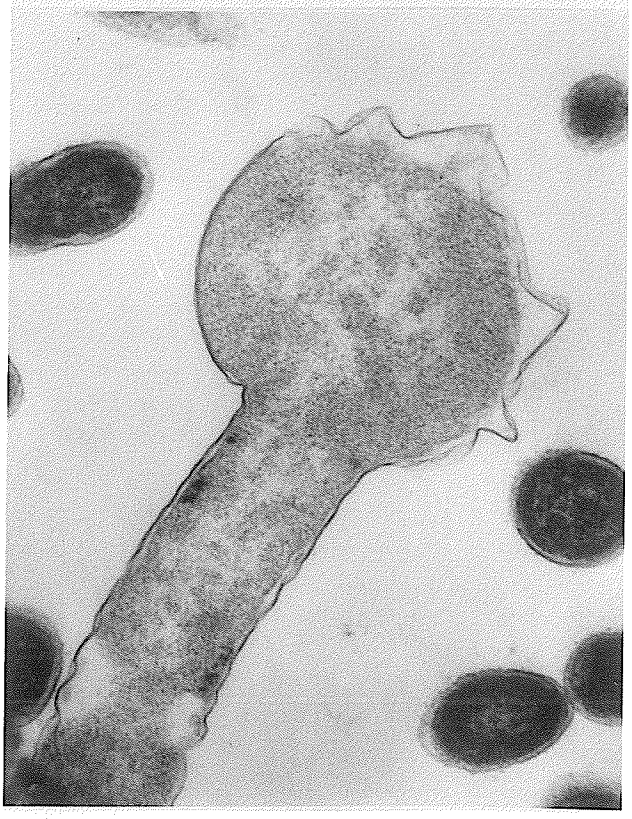
It is also suggested that the imbalance in protein synthesis can not only result in an overproduction of membrane but also in a decreased rate of activity of certain enzymes. This may be the result of a delay in: a) the time between initiation of chromosomic cycle and cell separation, or b) the time between termination of chromosomic cycle and cell separation, or c) the delay in the closing of the septum (Sargent, 1978). However, if the rate of the chromosic replication time increased,

Figure 12 and 13. Erwinia carotovora Grown in Minimal Medium with Maltotriose
Total Magnification (Microscopic and Photographic) is 30,000X

12 Black vesicles within elongated cell suggesting possible extra membrane structures

13 Whorl-like terminal structure





the appearance of new growth sites could account for this phenomenon. Clearly, additional information is necessary to understand this finding.

5. Minimum Inhibitory Concentration

Three different temperatures were tested to obtain a minimum inhibitory concentration in liquid medium. At 27°C and at 10°C, no total inhibitory effect was found at any level of antibiotic. However, at 4°C, total inhibition was observed at 6000 μgm/ml of 3G after 8 days (Table 4). The fact that the total inhibition was observed at this temperature is very important, since 4°C is the temperature used in storage of potatoes, where Erwinia carotovora is responsible for great losses. The significance of this low temperature effect is not clear.

Conclusion

This study established that the trisaccharide maltotriose does indeed inhibit Erwinia carotovora both in-vitro and also in-vivo. Although it was observed that in liquid and solid media the inhibition was partial, a minimum inhibitory concentration of 6000 μgm/ml was found in-vitro when the sample was incubated at 4°C for 8 days.

Table 4

Changes in Absorbance when Erwinia carotovora was Grown in Minimal Medium with Different Concentrations of Maltotriose

3G Concentration	Temperature 27°C	10°C	4°C
10000	*	0.337	0.220
8000		0.414	0.220
6000		0.419	0.220**
5000		0.444	0.358
4000		0.459	0.362
2000		0.455	0.378
1000		0.465	0.384
250		0.471	0.404
125		0.500	0.448
0		0.540	0.475

All readings (at 373 nm) were taken at 8 days after inoculation.

* At time of the first readings, overgrowth had occurred and absorbance readings were not recorded.

** End point i.e. MIC of 6000 μgm/ml.

The biochemical tests of Erwinia carotovora are still not totally clear since two of the nine results obtained for these kind of tests differ from the ones of Lelliot (1974). The fact that the values of pH were different for solid medium and for liquid medium suggest a physiological variability of the microorganism. This may depend upon certain conditions (i.e. water availability) and therefore, may be a possible explanation for the diverse taxonomical observations reported by many researchers.

Until now, no real explanation has been found to explain why a simple molecule like maltotriose contains inhibitory effects against Erwinia carotovora and not against other gram positive or gram negative microorganisms (Tanaka et al., 1975). It is suggested that the reason for the unique inhibitory effect of 3G on E. carotovora could also be related to the explanation for the variability of reactions of the microorganism under certain conditions. Another possibility to understand this variability of responses could be a study of other, well known, glucosidic antibiotic on Erwinia carotovora. Recognizing that 3G is commonly found in food systems (i.e. syrups, malt liquors, various fermentation products), it would seem that this compound

is not foreign to our food products.

More research is required to determine if indeed, 3G affects the balance of protein synthesis in Erwinia. It would be interesting to determine if 3G was responsible for the delay in closing of septum, since long cells divided at the latest stages of the cycle. Cell division seems to occur in the treated cells (Fig. 11c) suggesting that septum formation was not inhibited but the function of closing the septum was delayed. It would also be of interest to investigate the effect of 3G on the pectolytic activity of Erwinia carotovora, since these enzymes are responsible for soft rot production of plant tissue. Finally, it is suggested that a somewhat remote possibility of the action of 3G might be an amidation reaction peculiar to Erwinia. Labelled reactive groups might provide a possible study route into this area.

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Appendix 1

Effect of 3G in Minimal Medium

Time (h)	Absorbance at 373 nm	
	With 3G	Without 3G
17	0.832	0.839
20	1.167	1.214
23	1.205	1.405
27	1.374	1.547
31	1.535	1.692
41	1.575	1.718
45	1.573	1.728
47	1.544	1.729
52	1.566	1.726
63	1.487	1.762

Partial inhibition = 31.26%