AN ABSTRACT OF THE THESIS OF

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Title: EFFECT OF TEMPERATURE CYCLING ON THE

PRODUCTION OF AFLATOXIN BY ASPERGILLUS FLAVUS.

Abstract approved: _____

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The effect of cycling temperatures on production of aflatoxin by Aspergillus flavus (V3734-10) when grown upon various substrates was studied. The parameters of temperature and time were selected to simulate environmental conditions in Oregon during harvest of filberts and walnuts. The heat input required for aflatoxin synthesis in terms of degree hours per day were calculated and may be used as an index to predict potential danger of aflatoxin contamination. Conditions which generated less than 208° hours per day did not receive sufficient heat to induce growth and metabolism. When heat input ranged between 208 and 270° hours per day, growth and metabolism occurred with the development of a yellow pigment. There was not sufficient heat input, however, to induce the idiophase, sporulation and subsequent aflatoxin synthesis. Above 270° hours per day the

culture entered the idiophase, sporulation occurred and aflatoxin was produced.

The heat requirements for aflatoxin production was compared to the degree hours produced in the orchards by prevailing weather conditions at the time of harvest. The nuts are most susceptible to contamination and fungal growth during harvest when the nuts are damp and on the ground. During October and November, heat input is too low for aflatoxin production. From mid September to October heat input may be sufficient for aflatoxin production but at this time most walnuts and filberts are still tree borne and moisture in the environment is likely to be a limiting factor.

A. flavus was found to be a poor competitor when grown with the natural fungal flora isolated from moldy nut meats. On rice and nutmeat substrates, A. flavus was completely overgrown by members of the natural flora; even when A. flavus spores were present in superior numbers. Aflatoxin was not detected in the substrates of these cultures.

Effect of Temperature Cycling on the Production
of Aflatoxin by <u>Aspergillus</u> <u>flavus</u>

by

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INTRODUCTION

Disease caused by aflatoxin was first discovered in 1960 when
a series of outbreaks of unexplained death occurred in turkey flocks
in England (Blount, 1961). The syndrome observed in more than
100,000 birds became known as "Turkey X" disease. Reports of
similar outbreaks in ducklings and chickens (Asplin and Carnaghan,
1961), swine (Loosmore and Harding, 1961) and young cattle (Loosmore
and Markson, 1961) soon followed. The common factor to these out-
breaks was discovered to be peanut meal imported from Brazil.

The intensive investigations which followed uncovered the
following important information concerning aflatoxicosis:

1. The toxic agent was heretofore unreported.

2. The toxic agent was produced by mold Aspergillus flavus.

3. Toxic samples were recovered from at least 14 peanut-
   producing countries besides Brazil, indicating contami-
   nation was not restricted to a single source.

4. Significant levels of aflatoxin have been found in oilseed
   crops, grains, pulses and other food staples (Allcroft and

Carnaghan, 1963; Loosmore et al., 1964; Wogan, 1968).

5. Recent research has demonstrated presence of aflatoxin in milk products and fresh processed meat. In these products, aflatoxin had its origin in contaminated feed consumed by the animal (Keyl and Booth, 1971; McKinney et al., 1973).

6. In relatively large doses the toxin produces acute toxicity in several test animals. It also has carcinogenic effects on some test animals at very low levels.

With so many avenues open for aflatoxin to reach man through his food, it has become imperative to identify the uncommon as well as the common foods on which toxins may develop. It is also essential to gain an understanding of the environmental parameters which permit aflatoxin production. Toxin production on peanuts requires an ambient temperature of 10° to 45^oC, with an optimum between 20^oC - 35^oC and a water activity (Aw) of 0.75 or greater. The equilibrium moisture content at this level of water activity is about 9 percent on a wet weight basis (Austwick and Ayerst, 1963). Early work with peanuts showed aflatoxin can occur only before post harvest drying reduces the moisture below the critical level. The most critical stage is probably immediately following harvest while the nuts are still at a high moisture content. If drying is delayed by weather conditions or poor harvesting practices, the risk of mold

growth and aflatoxin production becomes great.

Two commercial nut crops are produced in Oregon, the filbert (Cerylus avellana) and the walnut (Juglans regia). These nuts are grown on trees, but because of the present harvesting conditions and the high moisture conditions prevailing during harvest time, they may have similar exposure to A. flavus invasion as ground nuts. Both nuts fall from the tree, usually free of the husk in early October after the rainy season has begun. The nuts may remain upon the ground, in a damp condition, up to two weeks and in extreme cases, over a month.

Under favorable conditions of temperature and moisture, both filbert and walnut meats are capable of supporting growth of A. flavus and production of aflatoxin. Extensive examination of the 1969 Oregon walnut crop and the 1972 Oregon filbert crop, however, did not reveal the presence of aflatoxin (Krumperman, 1973). Samples of Oregon nuts examined for aflatoxin by the Federal Regulatory Agency on a routine basis since 1969 have also proven negative.

Since this problem deals with a biological system the subtle factors relating to the synthesis of aflatoxin in a natural environment may be extremely difficult to ascertain. There are however four parameters that are obvious and can be measured with some degree of ease. These parameters are temperature, moisture, substrate and the natural fungal microflora. Under conditions of harvest in

Oregon the moisture may be assumed to be fairly consistent and non-limiting. The substrate, which may vary within certain limits, may also be assumed as a constant. The remaining two parameters, temperature and natural fungal microflora are much more variable and are the subject of this study.

Much of the work done in finding the essential growth parameters has been done in the laboratory under controlled temperatures and with pure cultures. While important information has been obtained under these well defined conditions, not all these conditions may be present in a natural habitat, particularly under harvest conditions of tree nuts in Oregon. Laboratory experiments were conducted simulating diruanal and nocturnal conditions of temperature ranging from 35° to $80^{\circ}F$ and saturated humidities. The degree-hours for aflatoxin development was calculated from the temperatures of incubation and the time required for the toxin to be produced. The effects of mixed flora on aflatoxin production were also included in the study, six fungi common to both filberts and walnuts were used.

REVIEW OF LITERATURE

Temperature Parameters for Production of Aflatoxin

Aspergillus flavus has been classified by Semeniuk (1954) as a mesophilic fungi growing with a temperature minimum between 6° - 8°C an optimum between 36° - 38°C and maximum between 44° - 46°C. Aflatoxin production has similar temperature requirements but is somewhat more restrictive. The strain of A. flavus and the substrate will, however, cause considerable variation in aflatoxin production. Generally, aflatoxin will be synthesized between the temperatures of 10° and 42°C with an optimum between 20° - 35°C (Diener and Davis, 1966a, 1968b; Eldridge, 1968; Schindler et al., 1967; Schroeder and Hein, 1967; Van Walbeek et al., 1969; Trenk and Hartman, 1970).

Schroeder and Hein (1967) have shown a time-temperature relationship in the production of aflatoxin on rough rice, cottonseed and peanuts. After 48 hours incubation, cultures held at 35°C produced 4 times the toxin as cultures held at 30°C and 22 times that of cultures held at 25°C. As long as the temperature is within limits for aflatoxin development, the relationship is valid. After the temperature exceeds 43°C all aflatoxin production ceases. As the temperature is lowered below the optimum, the time with which aflatoxin first appears is greatly extended and the time-temperature relationship

becomes nonlinear.

Effects of Temperature on Aflatoxin Production in Certain Food Products

Aflatoxin Production in Nut Crops

Nuts were the first food material implicated in aflatoxin poisoning. In 1961, Brazilian groundnuts were found to be a supporting medium for A. flavus. Subsequent studies found A. flavus produced a toxin which has been demonstrated as the causative agent of Turkey X disease in Great Britain (Blount, 1961; Sargent et al., 1961). Since that time almost all varieties of nuts have been shown as potential means by which aflatoxin may reach man and animals (Frank, 1966; Van Walbeek, 1968; Lillard et al., 1970; Krumperman, 1973).

Many of the nuts are raised and harvested in warm moist climates, and often allowed to lay on the ground during harvest time. Both conditions are highly conducive to growth of A. flavus (Bampton, 1963; Diener and Davis, 1969). Under such conditions the shell is the primary barrier preventing invasion of A. flavus (McDonald and Harkness, 1963; Ashworth and Langely, 1964; Schroeder and Ashworth, 1965; Diener and Davis, 1968a). The type and condition of the shell is of great importance in retarding development of mycotoxins on nuts. Peanuts and walnuts both have loose open shells and

are more susceptible to invasion than nuts such as peacans, filberts

and brazil nuts. Schroeder and Ashworth (1965), studying the rela-

tionship between the occurrence of aflatoxin and shell conditions of

spanish peanuts, reported aflatoxins could be detected only in kernels

with broken pods.

Because aflatoxin occurs so frequently on peanuts, they have

been studied more extensively than any other food product. The

conditions under which peanuts are harvested are so conducive to

growth of A. flavus, that nuts free from toxin when taken from the

ground, may become highly toxic 48 hours later (Bampton, 1963;

Ashworth et al., 1965).

Diener and Davis (1967) have outlined the parameters for

growth of A. flavus and production of aflatoxin on peanuts. They

innoculated sound mature kernels, broken mature kernels, immature

kernels and unshelled early runner peanuts with A. flavus ala-6.

Their results indicate a minimum relative humidity of 80 percent, a

minimum temperature of 13°C and a maximum temperature of 41.5°C

as limits for aflatoxin production. Similar results have been reported

by Bampton (1963) and Schroeder and Hein (1967).

Tree nuts may also be grown in environments where tempera-

ture and humidity are within the optimum range for A. flavus. Many

of these nuts, such as Brazilnut (Frank, 1966; Van Walbeek, 1968),

coconut (Frank, 1966), Pecans (Lillard et al., 1970), almonds,

filberts, and walnuts (Frank, 1966; Krumperman, 1973) have been shown to support aflatoxin generation. The parameters for aflatoxin development on tree nuts are not as well defined as with groundnuts. It is believed that contamination occurs primarily during harvest time when the nuts fall from the tree, although there is some indication insect damage may allow A. flavus to reach the nut meat while on the tree. Many of the tree nuts have a hard impervious shell which is a barrier to A. flavus invasion. When contamination does occur, it usually follows damage to the shell by insects, insect larva or other mechanical damage. (Krumperman, 1973).

Aflatoxin Production in Cereal Grains and Oil Seeds

Cereal grains and oilseed plants have been studied as substrates for aflatoxin production for two primary reasons; first many of these products are grown under conditions favorable for aflatoxin production and secondly they have been shown to be excellent substrates for A. flavus and toxin production. Because they are excellent substrates they have been used extensively in the laboratory. The frequency with which these products are used, necessitates a thorough knowledge of each product and its relationship to aflatoxin production.

When A. flavus is grown on oilseeds or cereal grains the effects of temperature on aflatoxin production are similar to those

observed with peanuts. Ashworth et al. (1969) reported maximum production of aflatoxin on cottonseed between 20° - 30°C. Eldridge (1968) reported aflatoxin developed most rapidly within the range of 25° - 35°C on a soybean substrate with temperature limits of 13°C and 40°C and no aflatoxin found at either 10°C or 42°C. Trenk and Hartman (1968) found temperature optimum for aflatoxin production on corn to be slightly higher than soybean. They indicated maximum toxin production between 30° - 35°C. These same investigators working with cold temperatures, found aflatoxin in only 1 of 105 samples of corn at 13°C. Epstein et al. (1970) demonstrated aflatoxin production on corn after 140 hours incubation at 15.6°C.

Rice is a widely used substrate in the laboratory because it provides the nutrients needed for luxuriant growth of A. flavus and production of aflatoxin, particularly B_1 (Stubblefield et al., 1967; Ashworth et al., 1969). Rice is also preferred because it contains little material which interferes with aflatoxin analysis. The optimum temperature range for aflatoxin production is the same as other substrates. As the temperature decreases from the optimum, aflatoxin production decreases and the time requirement becomes greater. This trend continues until the temperature reaches between 10 and 11°C the point production stops (Schroeder and Hein, 1967).

Other grains and oilseeds are also capable of supporting growth of A. flavus and aflatoxin production (Diener and Davis, 1966b;

Hesseltine et al., 1966; Stubblefield et al., 1967). However, most oilseeds and grains are not grown under conditions where natural contamination normally takes place. Shotwell et al. (1969a, 1969b) made a survey of wheat, grain sorghum, oats, corn, and soybeans and reported small amounts of aflatoxin, but only with poorer grades. They felt normal grading of these crops removed any hazard to man or animal.

Effect of Variable Humidity and Temperatures on Production of Aflatoxin

Aflatoxin Production Under Extreme Temperatures

The production of aflatoxin has been shown to closely follow mycellium growth until temperatures reach the range of 43° to 45°C (Diener and Davis, 1967, 1968b). Above these temperatures aflatoxin is not produced (Diener and Davis, 1966a, 1970; Dickens and Pattee, 1966; Schindler et al., 1967; Schroeder and Hein, 1968). Burrell (1964) has shown temperature to have an inhibitory effect when raised to 50°C for 2 to 4 hours, and then lowered to optimum again. The inhibition lasted up to 24 hours.

Unlike the maximum temperature, a lower limit is dependent upon substrate, time and strain of A. flavus used. This has been shown by several investigators using A. flavus strain V3734/10. Van Walbeek (1969) found aflatoxin on dextrose agar after 3 weeks and on

mycolgical broth with 0.5% yeast extract after 1 week at 7.5°C.

Sorenson et al. (1967), using rice as a substrate could not find any

aflatoxin at 8°C but fond toxin at 11°C after 3 weeks. Bullerman et al.

(1969a) reports aflatoxin on rice after 56 days at 10°C. Other workers

using different strains and substrates have reported a variety of

minimum temperatures, Schindler et al. (1967) working with isolates

M93 and M122, on wort agar, was unable to measure aflatoxin at 2°

or 7°C. Diener and Davis (1967) showed 12.5°C to be the minimum

temperature for aflatoxin production on peanuts by A. flavus ala-6.

Regardless of strain or substrate there is a consistant increase in the

time required for aflatoxin production and a decrease in production as

the temperature drops below the optimum.

Aflatoxin Production Under Fluctuating Temperatures

Most aflatoxin studies have been conducted under constant

temperatures. While these studies have provided considerable

information, aflatoxin generation in a natural environment often

occurs under cyclothermic conditions. Schroeder and Hein (1968)

conducted experiments simulating cyclothermic conditions using

Czapek agar. Temperatures were flucuated on a 12 hour cycle but

maintained a 25°C average for each 24 hour period. Their results

indicate little effect on mycellium growth at the low temperatures

used. At higher temperatures, however, growth declined rapidly.

Dickens and Pattee (1966 demonstrated cool weather even at high humidity will stop aflatoxin production on inoculated peanuts. These results appear to contradict those of Schroeder and Hein. Jackson (1965) in discussing his findings with peanuts in Georgia, states, "although conditions of higher moisture and low radiation in windrow II seems more conducive to fungus growth, the lower prevailing temperatures and shorter time in the soil were apparently the principle factors resulting in reduced kernel invasion". The foregoing information indicates cycling temperatures will slow down invasion by A. flavus and subsequent reduction of aflatoxin.

Aflatoxin Production with Variation in Humidity and Temperature

Austwick and Ayerst (1963) have reported moisture and temperature are the most important factors in the growth of A. flavus and generation of aflatoxin. Snow (1949) has shown the amount of moisture needed for fungi vary between species and strains and reported pathogenic strains of fungi normally require more moisture to sporulate than nonpathogenic strains. He found A. repens will sporulate at 76 percent relative humidity while A. echinulates will sporulate at 62 to 66 percent relative humidity after 2 years incubation. A. flavus, which is considered pathogenic, requires relative humidity conditions above 80 percent for mycellial growth and 84 percent before sporulation occurs (Bampton, 1963; Diener

and Davis, 1967, 1970; Sanders, et al., 1968).

Austwick and Ayerst (1963) found it necessary to vary the relative humidity inversely to changes in temperature to attain the same growth per day. One tenth millimeter growth per day was obtained at 15°C and 95 percent relative humidity, 18°C and 90 percent relative humidity, and at 20°C and 85 percent relative humidity. This pattern is continued until temperature and relative humidity are 30°C and 80 percent. Above 35° moisture requirements approach 95 percent relative humidity.

The moisture required for mold to sporulate is also directly related to the temperature of incubation. When the temperature is within the optimum range, moisture requirements will be minimum (Diener and Davis, 1968b). Below 20°C the relative humidity requirements for production of aflatoxin are from 97 to 99 percent (Diener and Davis, 1967). From 20°C to 30°C a minimum of 83 to 85 percent relative humidity is required (Diener and Davis, 1967, 1968b, 1970). At the optimum range of 30°C to 35°C relative humidity may be as low as 80 percent (Bampton, 1968; Trenk and Hartman, 1970). Above 35°C the moisture requirements will again rise to 97 to 99 percent (Diener and Davis, 1967). Trenk and Hartman (1968, 1970) have shown when temperature is held constant between 24°C and 35°C and the moisture varied by 18 to 26 percent, aflatoxin production will decrease linearly. Sanders et al. (1968) found by varying the

relative humidity from 99 to 92 percent there was a 80 percent de-

crease in aflatoxin production. If the relative humidity was

decreased from 92 to 86 percent aflatoxin production was decreased

95 percent. Their cultures were incubated at $25^{\circ}C$.

MATERIALS AND METHODS

Media

Polished rice has been used in the laboratory because it is effective as a substrate in the production of aflatoxin (Shotwell et al., 1966). It also has minimal material that interferes with the detection of aflatoxin. For these reasons, it was used to establish temperatures of aflatoxin production. The rice was prepared by mixing 10 grams with 40 ml water and sterilized in the autoclave for 15 minutes at 120°C (15 psi). Walnut and filbert substrates were prepared by mixing 10 grams of nut meat with 10 ml of water and sterilized in the same manner as rice. Incubation temperatures were identical to those of rice.

Cultures

A strain of Aspergillus flavus, V3734/10 (NRRL 2999) was obtained from Dr. W. Van Walbeek, Food and Drug Directorate, Department of National Health and Welfare, Ottawa, Canada. This strain was selected for its prolific mycellial growth and production of all four aflatoxins at refrigeration temperatures (Van Walbeek et al., 1969). The six fungi used in competitive studies were isolated from culled filbert meats obtained from various processing

plants in Oregon. These fungi were identified by Dr. W. C. Denison,

Curator of Mycological Herberium, Oregon State University,

Corvallis, Oregon. Stock cultures were maintained on Czapek agar

slants and stored at 5°C. Spores of the parent cultures were trans-

ferred to fresh agar and allowed to grow at room temperature for 5

days prior to use. Czapek agar was prepared according to the

instructions given in Difco Manual (1971).

Table I. Fungi Isolated from Culled Filbert Nut Meats

Fungi	Designation
Pencillium chrysoganym	GR-1, GR-2
Aspergillus flavus[a]	GR-3
Aspergillus niger	BR-1, BL-1
Aspergillus flavipes	GO-1
Aspergillus fumigatus	2
Rhizopus	WCB

[a]This strain of A. flavus believed to be a laboratory
contamination. It is very similar to V3734/10. Earlier
mycotoxin tests failed to show its presence.

Preparation of Suspension

Spores of V3734/10 were harvested from several rapidly

growing Czapek agar cultures by washing the agar surface with

sterile buffer solution. Spores were collected by filtration and

vacuum dried under 30 inches vacuum at 40°C. The dried spores

were dispersed on sterile powdered cellulose to provide a uniform inoculum throughout the study. Twenty two milligrams of the spore-cellulose preparation were added to 99 ml sterile water, giving a suspension of approximately 1.5×10^7 spores/ml. Spores of the isolated fungi were harvested by washing the slants with sterile water. Further dilutions were made as needed to give a desired concentration.

Incubation Chambers

A growth chamber was constructed to simulate environmental diurnal and nocturnal temperatures. The chamber consisted of a 2.5 refrigerator model TA 25-OCWH, modified to sit horizontally and run continuously. The small chamber volume insured even temperature distribution.

Two 1000 watt heating elements, controlled by variable resistors were placed in the refrigerator and adjusted to allow a slow change of temperature. One element was designed to override the cooling unit and provide controlled temperature increase. The other element was set to be overridden by the refrigeration unit. This provided slow temperature decline.

Maximum and minimum temperatures were controlled by two Honeywell temperature controllers, type T675A. These were inter-

connected to give a constant temperature within $\pm$ 1.5°C.

A Daytona 24 hour time clock was used to control dirunal and nocturnal time sequencing, a Telethermometer (YSI Model 42 SF) connected to a L and NH recorder provided a daily record of the chamber temperature. Koller culture flasks (320 ml) fitted with a stainless stell screen which was supported to provide a 0.5 inch space between the screen and the bottom of the flask were used. The screen served as a support for the media while allowing sterile water to be placed on the bottom of the flask to maintain a high relative humidity throughout the experiment. These flasks were incubated in the growth chamber.

Other samples were placed in 125 ml Erlenmeyer flasks and incubated in a series of water baths and refrigerators set at specific temperatures. These samples were shifted manually between high and low temperatures on a 12 hour interval to simulate the effects of fluctuating temperatures.

Growth Conditions

All samples were incubated until the first signs of sporulation were observed, the flasks were then removed and tested for aflatoxin. The temperatures in the growth chamber were adjusted to provide a 8.5°C differential between the high and low temperature. The range of the experiments were from 24°C to 1.5°C. The time required for

the temperature to rise and fall in the growth chamber was controlled

to span a 3 hour period. This was to approach as close as possible

environmental conditions. The temperatures in the incubators ranged

from $26.5^{\circ}C$ to $1.5^{\circ}C$ and were set at differentials ranging from $1.5^{\circ}C$

to $25^{\circ}C$. Rise and fall rates were not controlled and were different

for each program. The time for the temperature to go from one

extreme to the other required from 1 hour to 2.5 hours depending

upon the temperature spread of the program.

Extraction Procedures

Extraction procedures used were modifications of those

reported by Lee (1965). Fifty milliliters of chloroform were added

to each rice culture flask and the contents shaken for 30 minutes on a

Ebaback rotary shaker to extract the aflatoxin from the substrate.

The liquid fraction was collected in a separatory funnel and allowed

to stand 5 minutes to separate the chloroform extract containing the

aflatoxin, was filtered through a Whatman 2V filter and collected in

an Erlenmeyer flask. The aflatoxin was concentrated by using a hot

plate to evaporate the chloroform from the flask to an approximate

volume of 2 ml. The residue was transferred to a 10 ml volumetric

flask and brought to volume with chloroform.

Extraction procedures used to remove aflatoxin from walnuts

and filberts were similar to those used for rice with the exception of

Table II. Temperature Ranges of the Growth Chamber and Incubator

INCUBATORS

Program	Temperature $^{\circ}$C		Temperature difference $^{\circ}$C
	Maximum	Minimum	
I	32		
II A	26.5		
B	26.5	7.0	19.5
C	26.5	4.5	22.0
D	26.5	1.5	25.0
III A	21		
B	21	7.0	14.0
C	21	4.5	16.5
D	21	1.5	19.5
IV A	15.5		
B	15.5	7.0	8.5
C	15.5	4.5	11.0
D	15.5	1.5	14.0
V A	10		
B	10	7.0	3.0
C	10	4.5	5.5
D	10	1.5	8.5
VI	7.0		
VII	4.5		

GROWTH CHAMBER

VIII	24	15.5	8.5
IX	18.5	10	8.5
X	15.5	7.0	8.5
XI	13.0	4.5	8.5

filtering. In this case, the liquid fraction was passed through a

silica gel column to separate the oil and water from the chloroform-

aflatoxin extract (Eppely et al., 1968).

Qualitive Determination of Aflatoxin

Thin layer chromatographic (TLC) plates made from MN
Silica Gel G-Hr "adjusted for aflatoxin separation" were used to
determine the presence of aflatoxin. The silica gel was obtained
from Brinkman Instruments, Westbury, New York. Plates were
spread to a 0.25 mm thickness, dried for 30 minutes, activated by
heating 1 hour at $105^{\circ}C$, then stored in a dessicator until needed.

A 5 ml aliquot of each sample was spotted on a plate using
disposable micropipettes. Standards of 10 ug/ml B_1G_1 and 5 ug/ml
B_2G_2 aflatoxins were also placed on the plate. The plates were
developed in an air tight chamber using a developing solution of
chloroform, acetone, and 2-propanal (825:150:25 v/v/v) as described
by Pons et al. (1966). After developing 1 hour, the plates were air
dried at room temperature and observed under long wave ultra violet
light.

Florescent color and Rf values of extracted compounds were
compared to aflatoxin standards for identification. No attempt was
made to quantitatively measure the aflatoxin present.

Reagents

Chloroform, acetone and 2-propanal which constituted the
developing solution used in the thin layer chromatographic procedure

were U.S.P. Grade. The chloroform used for aflatoxin extraction

from the culture substrate was Technical Grade.

RESULTS AND DISCUSSIONS

Effects of Reduced Constant Temperature on Aflatoxin Production

Samples of long grain rice were incubated at constant temper-
atures until spores were first observed; the cultures were then ex-
tracted and tested for the presence of aflatoxin. The time required
for generation of aflatoxin at constant temperatures are shown in
Table III and illustrated in Figure 1. At constant temperatures of
32°C and 26.5°C, spores appeared within 36 and 48 hours respect-
ively. At these temperatures the strain of A. flavus used produced
a dark green color from the spores and a substantial amount of
aflatoxin. As the temperature was decreased to 21°C and 15.5°C,
the time for sporulation increased while the number of spores and
the amount of aflatoxin decreased. A further decrease of tempera-
ture to 10°C caused sporulation and aflatoxin generation to stop.
Aflatoxin was not found at 10°C even with prolonged incubation.

Cyclo-Thermic Effects on Aflatoxin Production

Before toxin generation on agriculture commodities can be
controlled, conditions in the natural environment must be understood.
The environment is constantly changing in relation to temperature
making exact duplication in the laboratory impossible to achieve.

Table III. Time Required for Aflatoxin Production at Constant Temperatures

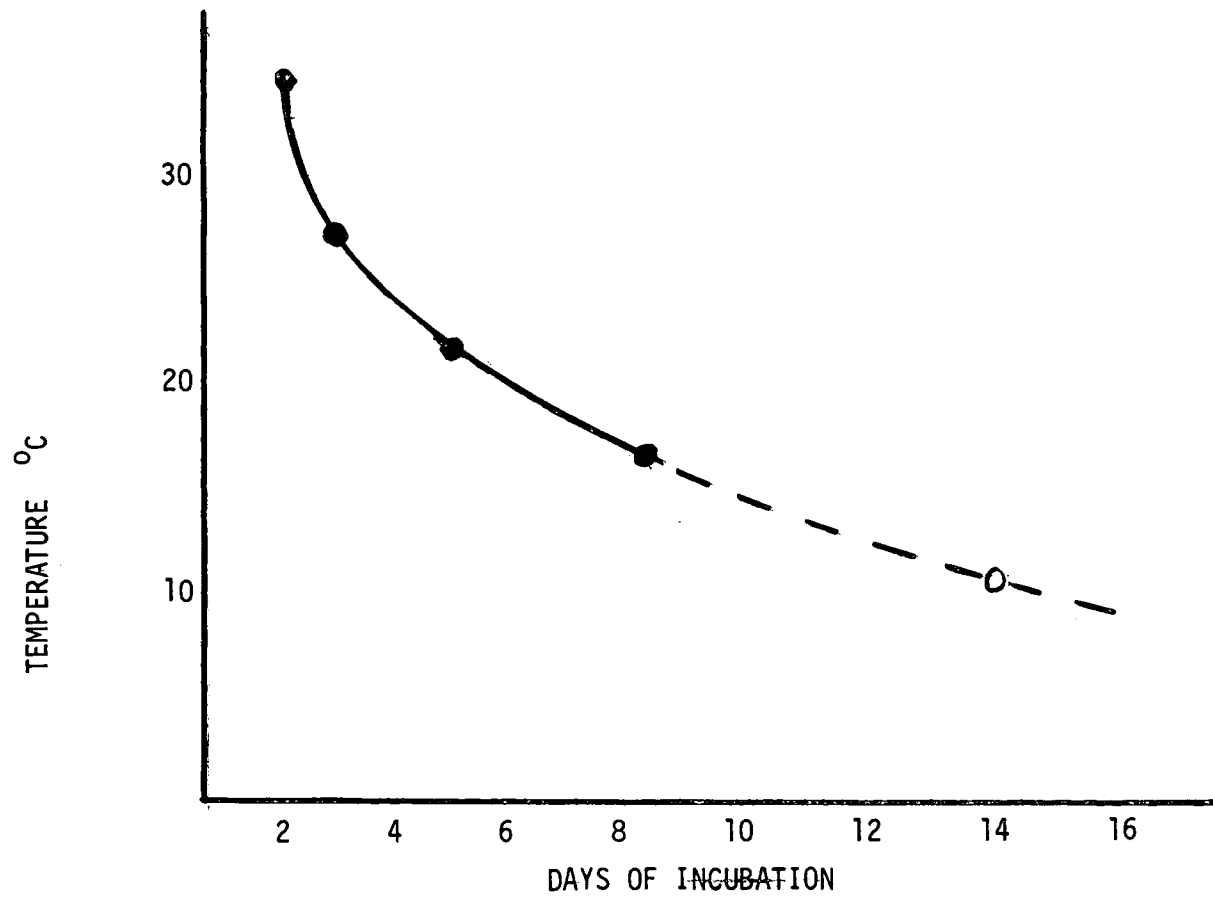| Temperature °C	Aflatoxin Produced
32	35 hours
26.5	46 hours
21	96 hours
15.5	8 days
10	No aflatoxin after 94 days
7.0	No aflatoxin after 104 days
4.5	No aflatoxin after 141 days

Figure 1. Days of Incubation Required for Aflatoxin Production Constant Temperatures

However, cycling between two temperatures can be accomplished and considerable information obtained.

The required thermal input for aflatoxin production under conditions of cycling temperature may be determined by using a modification of a method developed by crop scientists to predict harvest time. The thermal input is expressed in degree hours per 24 hour day and determined by plotting the temperature as it goes through a daily cycle. The minutes required for a change of $3^{\circ}C$ is multiplied by the average temperature over the 3° span. The total of these products, divided by 60, is an expression of degree hours developed during periods of temperature rise and fall. Degree hours for periods of constant temperature are similarly calculated. When added together these figures express the total degree hours per day and indicates the heat or energy input received by the culture over a 24 hour period. The degree hours for each of the programs are presented on Table IV. Daily cycles used in this study are illustrated in Figures 3 through 18.

Aflatoxin generation was affected very little by temperature cycling in those programs where the high temperature maxima resulted in a heat input well above the minimum for sporulation. When A. flavus was subjected to program II, heavy sporulation was observed and a large amount of aflatoxin was produced even when wide differences between the minimum and maximum temperature

Table IV. Degree Hours Per Day and Days of Incubation Required for Aflatoxin Production in the Incubators

Program	Temperature ^{o}C		Days for Aflatoxin Generation	Degree Hours Per Day
	Maximum	Minimum		
I	32	32	1.5	768
II A	26.5	26.5	2	648
B	26.5	7.0	4	418.4
C	26.5	4.5	5	382
D	26.5	1.5	6	362.3
III A	21	21	4	504
B	21	7.0	8	331.1
C	21	4.5	12	308.6
D	21	1.5	15	269.7
IV A	15.5	15.5	8	373
B	15.5	7.0	No Aflatoxin in 68 days	267.1
C	15.5	4.5	" " " 68 "	238.4
D	15.5	1.5	" " " 68 "	219.6
V A	10	10	" " " 94 "	240
B	10	7.0	" " " 68 "	204.7
C	10	4.5	" " " 68 "	189.1
D	10	1.5	" " " 68 "	142.8
VI	7.0	7.0	" " "104 "	168
VII	4.5	4.5	" " "141 "	108

occurred.

Aflatoxin generation is more obviously affected by temperature cycling when the programmed temperature approached the minimum heat input for sporulation. Table IV shows $15.5^{\circ}C$ to be sufficient for aflatoxin to be produced. However, any fluctuation in temperature, as was done under program IV, resulted in the degree hours per day dropping below the minimum for aflatoxin generation. Cultures in program IV were incubated for 68 days without aflatoxin being produced. In contrast, cultures placed in programs III C and D, producing slightly over 270 degree hours per day, synthesized small quantities of aflatoxin after 12 to 15 days incubation.

Experimental data show 267.1 degree hours per day of program IV B to be below the minimum for aflatoxin production. Although the difference between 267.1 and 269.9 is small, the difference is significant in terms of aflatoxin generation for the strain of A. flavus used. At some point between these two limits the mechanism of toxin production shuts down. As the degree hours per day approached 269.9, incubation time for aflatoxin production increased to a maximum of 15 days. However, immediately below this heat level, no aflatoxin was detected. Incubation times extended from 68 to 141 days at the lower degree hours per day.

Conditions found in the growth chamber were closer approximations of environmental conditions than the incubators. However,

examination of comparable degree hours per day under both condi-
tions indicated the two methods of temperature cycling to be consis-
tent with each other. The growth chamber was used in four experi-
ments (programs VIII, IX, X and XI). The results of these tests are
shown in Table V.

Two of the growth chamber tests, programs VIII and X had
mechanical failure which gave apparent inconsistent results. How-
ever, when these circumstances are taken into account, the results
are comparable to other tests with similar heat input. Program
VIII was to be cycled between $24^{\circ}C$ and $15.5^{\circ}C$. The recorder used
during the experiment was not accurate enough for this work result-
ing in the temperature range and degree hours per day approaching
program II D. In both tests (II and VIII) the culture turned yellow,
produced spores and developed Simplar mycellium densities in the
same length of time.

Samples incubated under program X produced 278.8 degree
hours per day at temperatures of $15.5^{\circ}C$ and $7.0^{\circ}C$. The tempera-
ture for this program and the incubator program IV were the same,
however, because of the controlled rate of temperature rise and fall
the degree hours per day in the growth chamber was higher. Due to
a second mechanical malfunction, test X fell below 270 degree hours
per day during the first week of the experiment. After replacing
faulty equipment, spores were produced within 14 days. When the

Table V. Degree Hours Per Day and Days of Incubation Required for Aflatoxin Production in the Growth Chamber

Program	Temperature $^\circ$C		Days for Aflatoxin Generation	Degree Hours Per Day
	Maximum	Minimum		
VIII	24	15.5	6	472.9[a]
IX	18	10	8	342.3
X	15.5	7.0	21[b]	278.8
XI	13	4.5	No Aflatoxin in 85 days	208.9

[a] Because of inaccurate recorder, degree hours for day were not actually as high as shown.

[b] Faulty relay switch prevent this program to achieve 278.8 degree hours per day during the first week of the experiment.

results of this test and those of programs III C and D are compared,

it can be seen that 14 days is the expected time for aflatoxin to be

produced at 278.8 degree hours per day.

Program IX produced 342.3 degree hours per day and

developed aflatoxin after 8 days. Incubator programs III B and IV A

produced 331.1 and 373 degree hours per day respectively requiring

8 days for aflatoxin generation. The results of this test indicate

total degree hours per day, within the growth limits for A. flavus,

and not the rate with which the heat input is applied is a determining

factor in the production of aflatoxin.

Program XI was incubated for 85 days at 208.9 degree hours

per day with no sign of aflatoxin production. However, it did turn

yellow after 12 days indicating some metabolism did occur. These

results are the same as those of programs IV D and V A and B

which also turned yellow but failed to produce aflatoxin.

The 270 degree hours per day minimum determined for afla-

toxin generation under cycling temperature conditions is in agreement

with the previously established limits for constant temperatures. No

aflatoxin production was noted at $10^{\circ}C$ (240 degree hours per day) but

there was at $15.5^{\circ}C$ (373 degree hours per day). A constant tempera-

ture of $11.3^{\circ}C$ provides 271.2 degree hours per day which is suf-

ficient thermal input to synthesize aflatoxin. Cultures on rice held

at this temperature should produce spores and aflatoxin within 14 to 15

days.

From these results, several facts about <u>A</u>. <u>flavus</u> can be noted:

1. A minimum of 270 degree hours per day is required for aflatoxin production under cycling conditions. The degree hours must be attained within the temperature limits for growth of <u>A</u>. <u>flavus</u>.

2. At the minimum heat input, aflatoxin will be produced within 15 to 16 days.

3. Temperature fluctuations will have a greater inhibitory effect on the cultures as the degree hours per day approaches 270.

Effect of Cold Temperatures on Growth of <u>A</u>. <u>flavus</u>

As <u>A</u>. <u>flavus</u> grows and spreads across the substrate, regular and predictable changes can be seen on rice. Growth is first noted by a change in color of the rice from white to tan which continues to intensify and change to yellow. The yellow pigment appears almost simultaneously with spores and aflatoxin on samples incubated above 270 degree hours per day. As the mycellial mat develops, the yellow color becomes less obvious but can be easily extracted with the aflatoxin. There appears to be a correlation between the intensity of the yellow pigment and the amount of aflatoxin generated.

Samples incubated between 208 and 270 degree hours per day do not produce spores or aflatoxin, however, the yellow pigment does appear and it continues to increase in intensity becoming bright orange in color. The orange color remained on the rice until the experiment was terminated. Both the yellow and orange pigment of these cultures is more difficult to extract with chloroform.

Three levels of activity were observed with A. flavus in relation to thermal input. The first level was noted with cultures receiving less than 208 degree hours per day. No visible growth or metabolism could be detected and prolonged incubation caused no apparent change. The second level occurred between 208 and 270 degree hours per day. At this level of heat input, mycellial growth was initiated and a yellow pigment produced. Prolonged incubation at this level effected a change in the pigment color from yellow to orange. The thermal input is sufficient to initiate and sustain growth but not for sporulation and aflatoxin generation. The third stage occurs above 270 degree hours per day. At this level of thermal input, the culture enters into an idiophase, sporulation occurs and aflatoxin is generated. With sporulation a green color develops becoming the dominate color of the culture.

Effects of Cool Temperatures on Production of Aflatoxin

As shown above, visible changes on rice cultures stop when

heat input drops below 208 degree hours per day. However, there still may be some metabolic activity at lower levels of heat input. All tests performed under program III were incubated under identical conditions except for minimum temperature. Cultures from this program show evidence of activity during the cold temperature phase of the daily cycle. Results of conditions the three tests were subjected to under program III are reported in tables VI and VII. Table VI shows the conditions prevailing above 10°C, where A. flavus growth is known to occur. Table VII reports conditions below 10°C. While the length of time needed for incubation increases with lower minimum temperatures, other differences were minimal and do not account for the extra 7 days needed for aflatoxin production during test D.

In order to study the increased incubation time caused by cold temperatures, rice cultures were incubated at 32°C for 34 hours. The cultures were divided with half remaining at 32°C and designated as "a", while the other half, designated as "b" was placed at 1.5^{o}C for 2 hours and then returned to 32°C. Samples from both cultures "a" and "b", were removed from the incubator every 2 hours and changes in the cultures as well as aflatoxin production were compared. Development of the yellow pigment and sporulation on the "b" cultures lagged behind the "a" cultures. The quantity of aflatoxin produced by each culture was determined by observing aflatoxin spot

densities on developed TLC plates. Quantitative comparisons of aflatoxin extracts showed culture "a" produced more total toxin than culture "b". Although culture "b" was exposed to 1.5°C for only two hours, it lagged behind culture "a" in the quantity of aflatoxin by approximately 4 hours throughout the duration of the experiment (Figure 2).

Summarizing the data obtained from cold temperature incubation, it was determined that: (i) at 10°C metabolism may be detected visually by the development of yellow pigment. Aflatoxin is generated very rapidly when the cultures are placed at slightly warmer temperatures. (ii) Visible changes are not detected in the rice medium when incubated at 7.0°C. When warmed above 10°C these cultures generated aflatoxin on a time schedule commensurate with the heat input. (iii) No visible changes occur on rice cultures incubated at 1.5°C. If the heat input is raised above 270 degree hours per day, aflatoxin development will proceed but will require at least an additional 2 hours over the expected time of incubation.

Effect of Normal Microflora on A. flavus

Six fungi were isolated from moldy filbert nut meats taken from various packing plants in the Willamette Valley. The fungi isolated, with the exception of GR-3, are believed to represent the predominate fungal flora of Oregon nut meats. GR-3 was identified

as A. flavus, however, it is believed to be a laboratory contamination rather than among the normal flora. Previous attempts to isolate A. flavus from filbert and walnut meats have failed and mycotoxin tests on suspect nut meats have not shown the presence of aflatoxin. The isolates of the normal flora were subjected to cycled temperatures of program III and V. All samples were incubated until spores were observed. Isolate designated as "2" was isolated after this study was under way and therefore not included.

Two sets of experiments were performed with the natural isolates. The first to establish their temperature requirements for growth and sporulation. The results of this experiment is shown in Table VIII. All isolates were shown to have a greater tolerance and grew more vigorously at low and cycled temperatures than A. flavus. It is of significance to note that most of the natural isolates sporulated at a thermal input level of 142.8 degree hours per day, well below the 270 required for A. flavus to sporulate and the 208 degree hours per day required for A. flavus to grow.

The second experiment was to determine if A. flavus could grow competitively with these fungi and produce aflatoxin under temperature conditions simulating the natural environment. Spores of GR-2 were mixed with spores from A. flavus at ratios of 2:4, 3:4 and 4:4. Rice cultures were prepared with the mixed inoculum and incubated at cycling temperatures previously used. At all combina-

Table VI. Conditions That Prevailed Under Incubator Program III at Temperatures Above 10°C.

Test	Temperature °C		Degree Hours Per Day above 10°C	Hours Per Day Above 10°C	Days of Incubation
	Maximum	Minimum			
B	21	7.0	245.7	12.5	8
C	21	4.5	254	12.8	12
D	21	1.5	245.9	12.3	15

Table VII. Conditions That Prevailed Under Incubator Program III at Temperatures Below 10°C.

Test	Temperature °C		Degree Hours Per Day Below 10°C	Hours Per Day Below 10°C
	Maximum	Minimum		
B	21	7.0	89.1	11.5
C	21	4.5	55.3	11.2
D	21	1.5	24.9	11.7
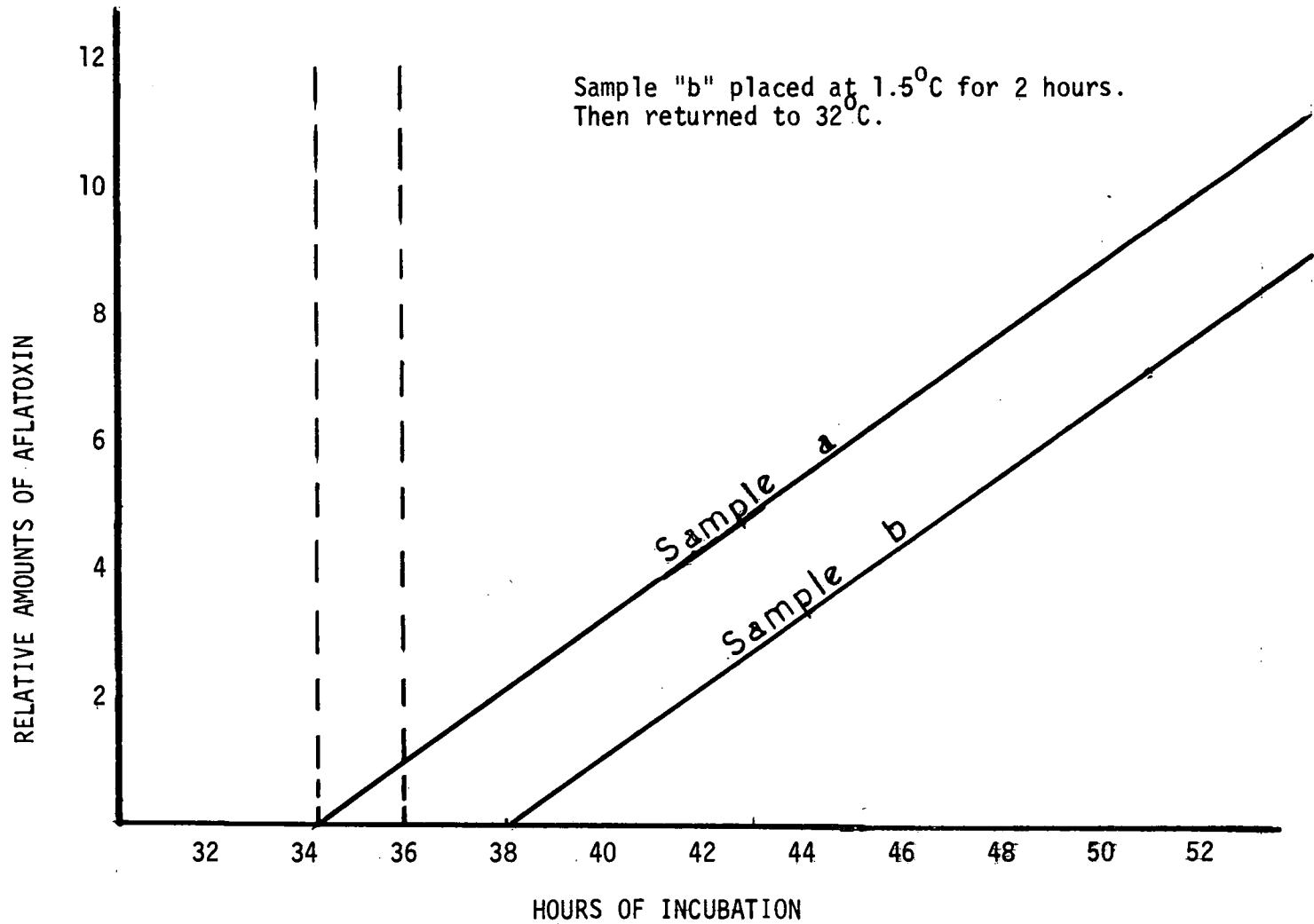
Figure 2.   Lag Period Produced by 2 Hour Incubation at 1.5° C

tions of temperature and spore ratios, GR-2 overgrew A. flavus and aflatoxin was not observed. These observations suggest A. flavus even when present in larger numbers, would have difficulty developing spores and aflatoxin among the natural flora which becomes established on nut meats under prevailing conditions at harvest time in Oregon.

Also germaine to this point is the contamination experienced on several occasions by Pseudomonas bacteria. These bacteria grew so well at cool temperatures, A. flavus was completely inhibited in their presence.

Comparison of Walnuts and Filberts to Rice as a Substrate for A. flavus

Chopped walnut meats were inoculated with A. flavus and incubated under the same conditions as the rice cultures. Similar studies were conducted on filberts but on a limited scale. These results are recorded in Table IX.

The walnut culture required the same minimum degree hours per day to sporulate and generated aflatoxin as the rice culture. This indicates the limiting factor for sporulation is a function of heat input and not the medium. The walnut substrate does, however, influence the length of time required before aflatoxin is generated. Rice culture produce aflatoxin in 15 days at approximately 270 degree hours

Table VIII. Days Required for Sporulation of Fungi Isolated from
Filbert Nut Meats.

| Culture | Temperature °C | | Days to Sporulate | | Degree Hours |
	Maximum	Minimum	Isolate	A. flavus	Per Day
GR-1	21	7.0	6	12	331.1
	21	4.5	8	13	308.6
	21	1.5	8	13	269.9
	10	7.0	11	NS[a]	204.7
	10	4.5	11	NS	189.1
	10	1.5	12	NS	142.8
GR-2	21	7.0	5	12	331.1
	21	4.5	5	13	308.6
	21	1.5	5	13	269.9
	10	7.0	8	NS	204.7
	10	4.5	8	NS	189.1
	10	1.5	8	NS	142.8
BL-1	21	7.0	8	12	331.1
	21	4.5	8	13	308.6
	21	1.5	8	13	269.9
	10	7.0	13	NS	204.7
	10	4.5	17	NS	189.1
	10	1.5	NS	NS	142.8
GO-1	21	7.0	8	12	331.1
	21	4.5	9	13	308.6
	21	1.5	9	13	269.9
	10	7.0	13	NS	204.7
	10	4.5	17	NS	189.1
	10	1.5	24	NS	142.8
WCB	21	7.0	9	12	331.1
	21	4.5	11	13	308.6
	21	1.5	12	13	269.9
	10	7.0	17	NS	204.7
	10	4.5	21	NS	189.1
	10	1.5	24	NS	142.8

a. No spores seen.

Table IX. Days Required for Aflatoxin Production on Rice, Walnuts and Filberts.

Temperature °C		Rice	Walnuts	Filberts	Degree hours Per Day
Maximum	Minimum				
27	27	2	3	2	648
27	7.0	4	5	4	418.4
27	4.5	5	6	5	382
27	1.5	6	9	6	362.3
21	21	4	5	4	504
21	7.0	8	17		331.1
21	4.5	12	19		308.6
21	1.5	15	27		269.7
15.5	15.5	8	19	12	373
15.5	7.0	NA[a]	NA		267.1
15.5	4.5	NA	NA		238.4
15.5	1.5	NA	NA		219.6
10	10	NA	NA		240

a. No aflatoxin produced.

per day, while walnuts required a minimum of 27 days at 270 degree hours per day.

The effect of cold temperatures on growth and sporulation of A. flavus on walnuts was more dramatic than on rice. At 27°C there is only a 24 hour difference in sporulation between the two substrates. As the heat input decreased and approached 270 degree hours per day the difference in time for sporulation between the two substrates almost doubled, requiring 27 days for walnut and 15 for the rice.

Because of the brown color of the walnuts, the presence of the yellow pigment could not be definitely be identified. A yellow pig-

ment is extracted from these cultures with chloroform, however it is generally darker than the one found on rice and could be a pigment from the nut. Mycellium growth is easier to identify on the walnut since it initially appears as a white fluffy mat. At early stages of growth, spore development does not appear to be as green and luxuriant on the walnut as on the rice. When incubated for extended periods of time, however, a dark green mycellial mat does develop.

Filberts were studied to a lesser extent than walnuts and rice. They appear to approach rice in the ability to support A. flavus at warmer temperatures. At cooler constant temperatures of $15.5^{o}C$, the incubation time becomes more extended. Mycellium growth on filberts is very easily detected. The initial phase is a fuzzy white growth protruding outward from the nut meat. As the incubation time increases the mycellium becomes a heavy white mat loosing much of its fluffiness. Spore development is dark green and similar in appearance to the rice culture after comparable incubation times.

A. flavus will grow only on the exposed meat of the walnut and filbert. Attempts to produce growth on the meat covering or shell alone have failed unless nitrogen is supplied.

Aflatoxin Production Under Prevailing Environmental Conditions
in Oregon during Nut Harvest

Because of the increased danger of infestation of nuts by A.

flavus during harvest time, examination of prevailing weather conditions at that time is necessary. Adequate meteorological records were available only in Corvallis and Roseburg weather stations. Since the weather environment in Corvallis is considered to be representative of prevailing weather conditions for the Eugene and Salem agricultural areas these records and the ones in Roseburg provided sufficient information for most of the area where nuts are cultivated in Oregon. Records for the north portion of the Willamette Valley were inadequate for the purpose of this study but meteorologist familiar with the area believe the temperatures to be lower than the Corvallis area. Bi-hourly temperatures prevailing during September, October and November for the years 1964 to 1972 at Corvallis, Oregon and 1966 to 1972 at Roseburg, Oregon were compiled and converted to degree hours per day above 10°C. Results of this compilation showing the days per month where the degree hours per day approached or exceeded 240 are recorded in Table X. From this data the most critical time when aflatoxin could be a problem is in September. Danger at this time is minimal since during early fall most nuts are still on the trees. It is believed contamination would most likely occur after the nut falls to the ground.

Table X. Days Per Month and Consecutive Days Where Degree Hours Per Day Exceeded 240

Corvallis, Oregon

	September		October		November	
Year	Days Per Month	Consecutive Days	Days Per Month	Consecutive Days	Days Per Month	Consecutive Days
1964	12	4	5	2	0	0
1965	17	7	6	5	3	3
1966	22	10	10	4	2	1
1967	26	11	7	4	0	0
1968	23	18	2	1	1	1
1969	23	7	1	1	1	1
1970	19	11	3	2	2	1
1971	20	15	5	3	0	0
1972	16	8	7	2	2	1

Roseburg, Oregon

Year	Days Per Month	Consecutive Days	Days Per Month	Consecutive Days
1966	17	17	10	6
1967	No Data		No Data	
1968	No Data		No Data	
1969	7	4	0	0
1970	2	1	No Data	
1971	0	0	0	0
1972	9	7	0	0

SUMMARY AND CONCLUSIONS

The effect of cycling temperatures on Aspergillus flavus and aflatoxin production from cultures grown on rice, walnuts and filberts may be summarized as follows:

1. Regardless of length of incubation, aflatoxin was not found on any culture receiving less than 270 degree hours per day.

2. The effect of cyclo-thermic conditions on A. flavus growth becomes less obvious as degree hours per day increase.

3. Regardless of how the heat is applied, incubation time will be the same for all cultures receiving similar degree hours per day, providing temperatures are within growth range for A. flavus, and all other parameters for growth are optimal.

4. A. flavus has three phases of development:

   a. Below 208 degree hours per day, activity for growth is not detectable.

   b. Between 208 and 270 degree hours per day a yellow pigment develops indicating growth. Aflatoxin will not develop in this range of heat input.

   c. Above 270 degree hours per day, spores develop and aflatoxin is produced.

5. _A. flavus_ is less adapted to cool environmental conditions than most other natural flora of the nut meat. In a cool environment _A. flavus_ is less competitive and will usually be overgrown.

6. While both filbert and walnut meat will support _A. flavus_ growth, the incubation times for aflatoxin generation is quite different for each. At warm temperatures, walnuts require slightly longer incubation periods than do filberts. _A. flavus_ produces aflatoxin on filberts at about the same rate as rice. When exposed to cool temperatures, toxin production for cultures on walnuts and filberts slows down more rapidly than rice cultures.

7. If contamination with _A. flavus_ occurs after the nut falls to the ground, aflatoxin production on Oregon nuts is not likely to occur. The degree hours per day developed during the time of year when nuts are on the ground is too low to allow _A. flavus_ to sporulate.

8. If contamination by _A. flavus_ occurs while the nuts are on the tree, aflatoxin may be produced. The degree hours per day developed in the Oregon Willamette Valley during the summer and early fall are sufficient for growth of _A. flavus_.

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APPENDIX
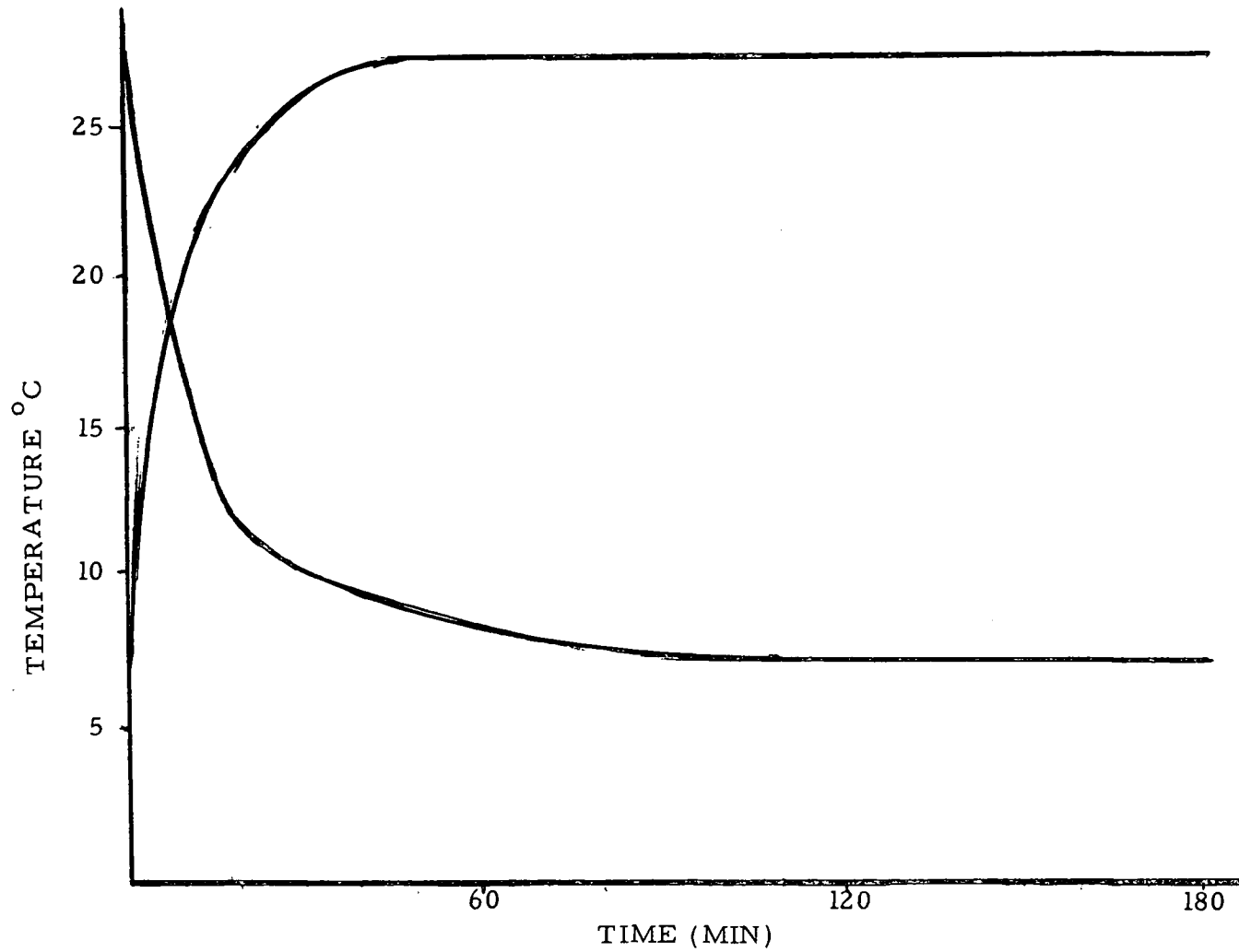
Figure 3. Temperature Change Rate in Incubator Under Program II B (27°C-4.5°C)
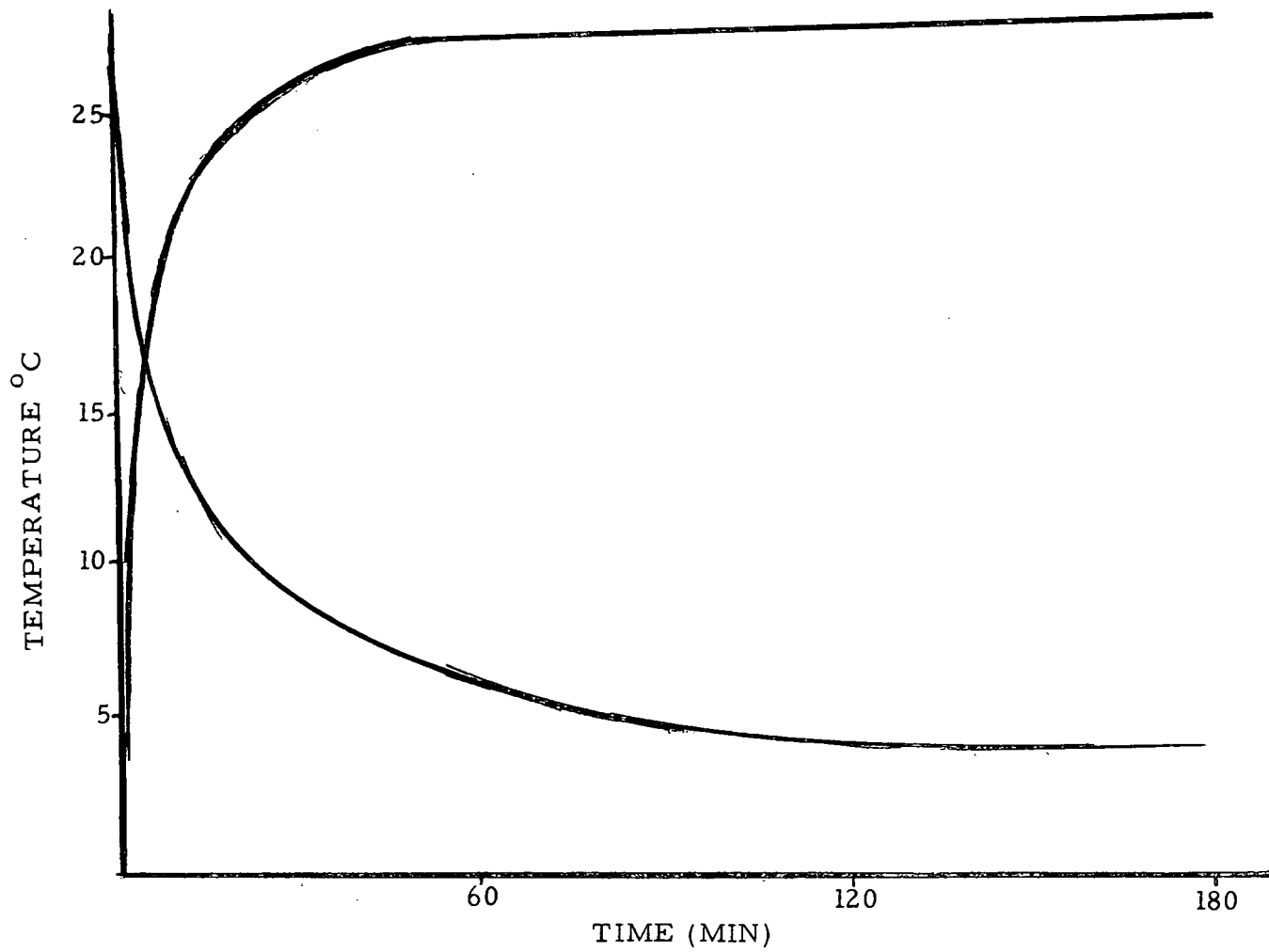
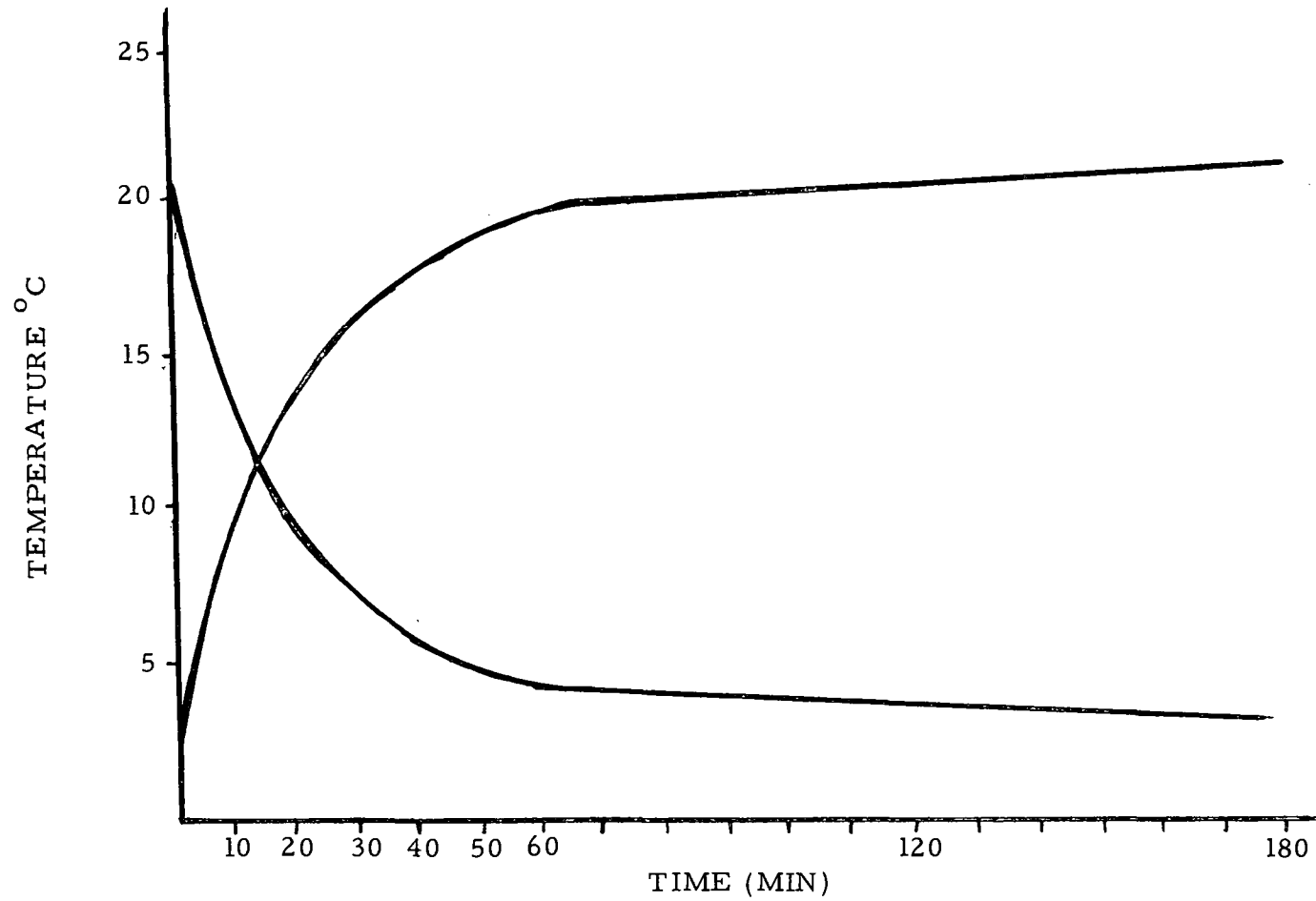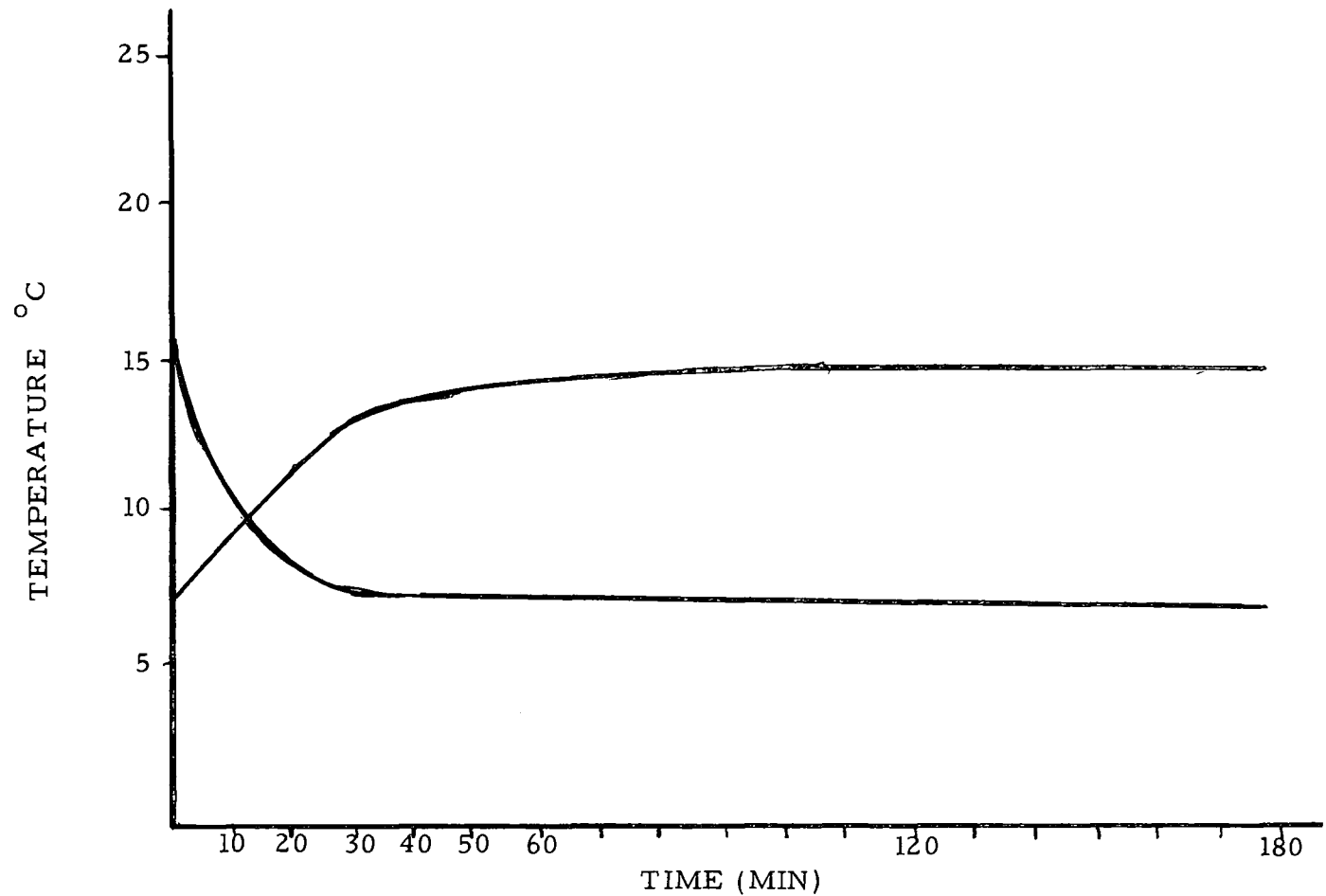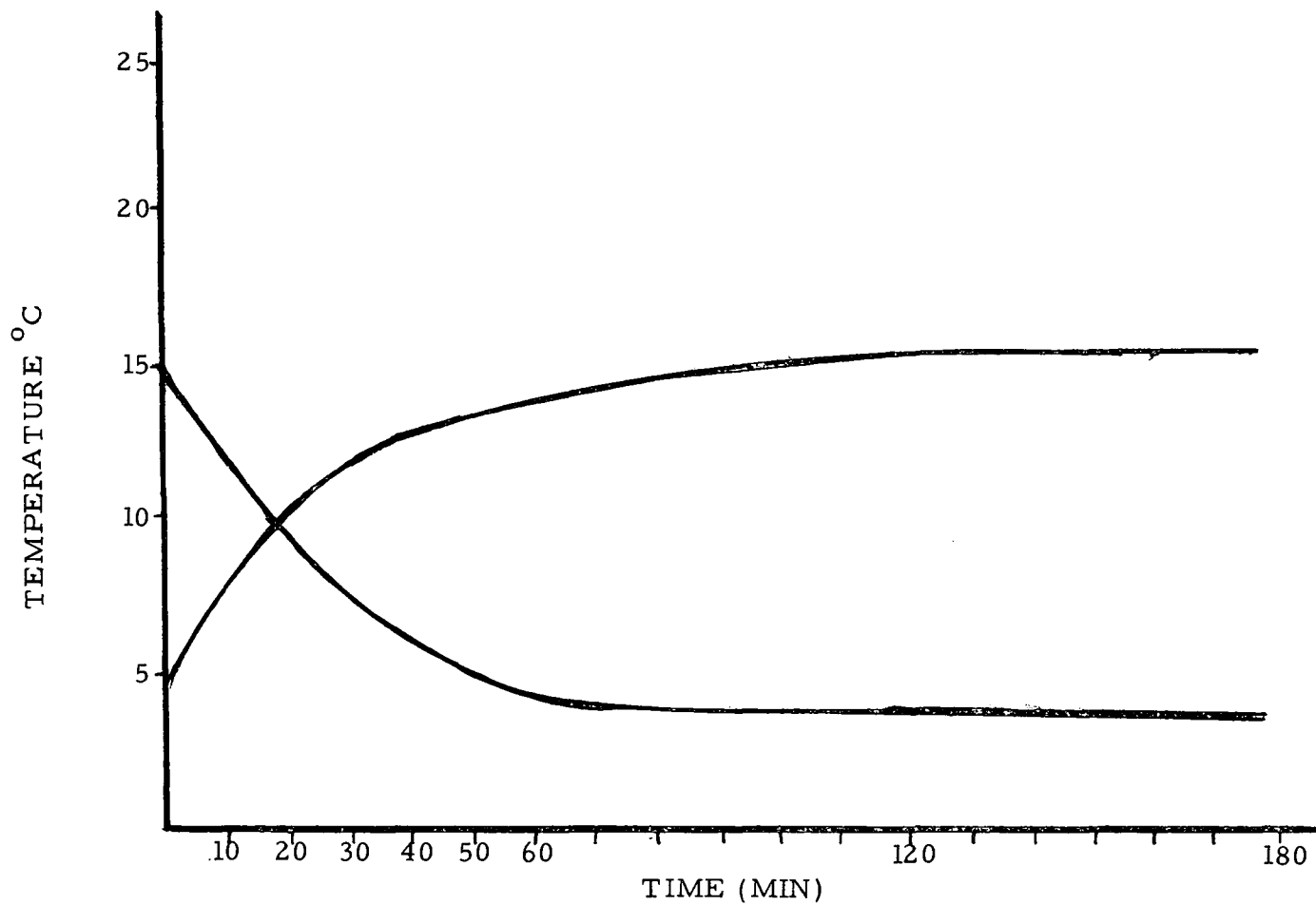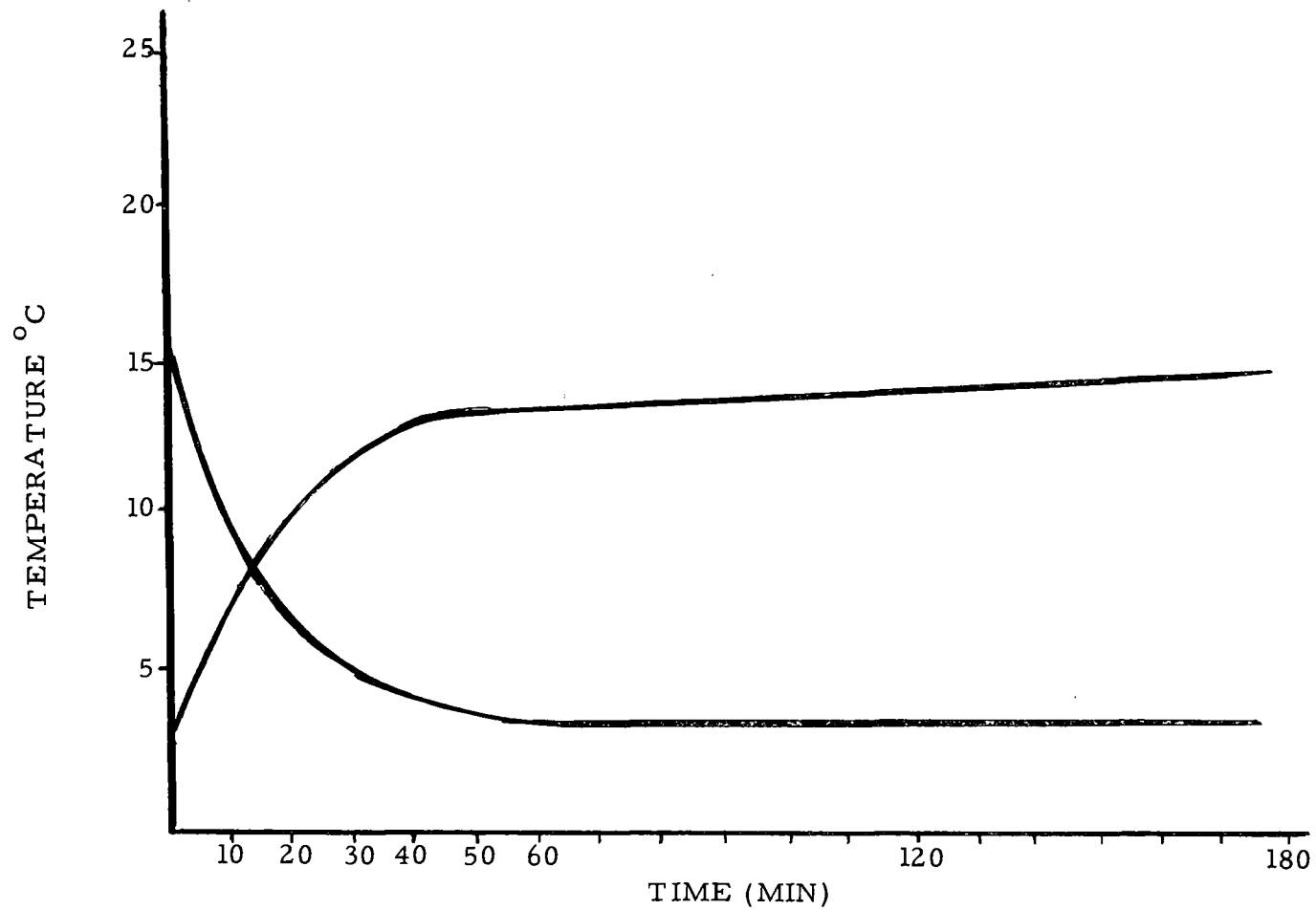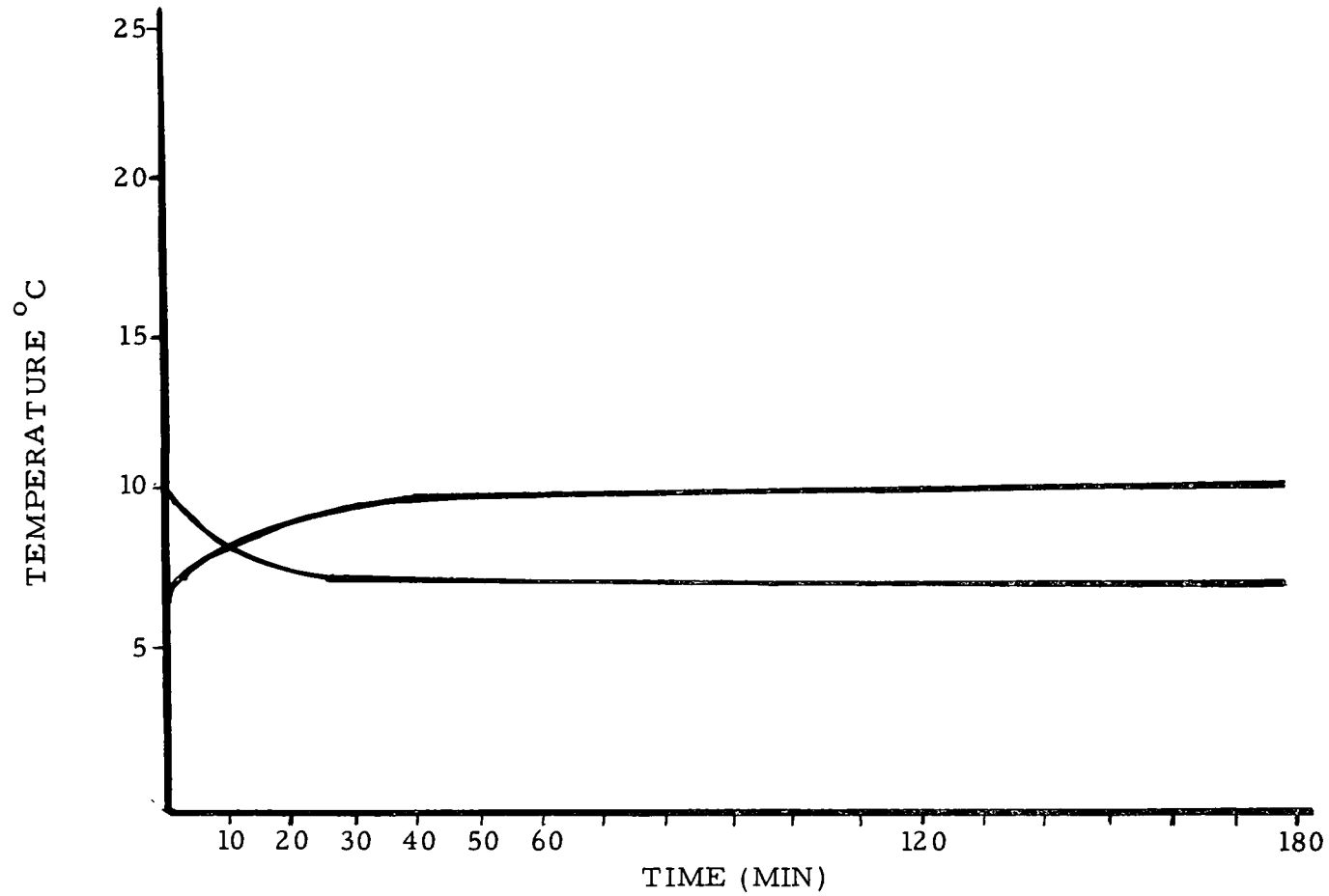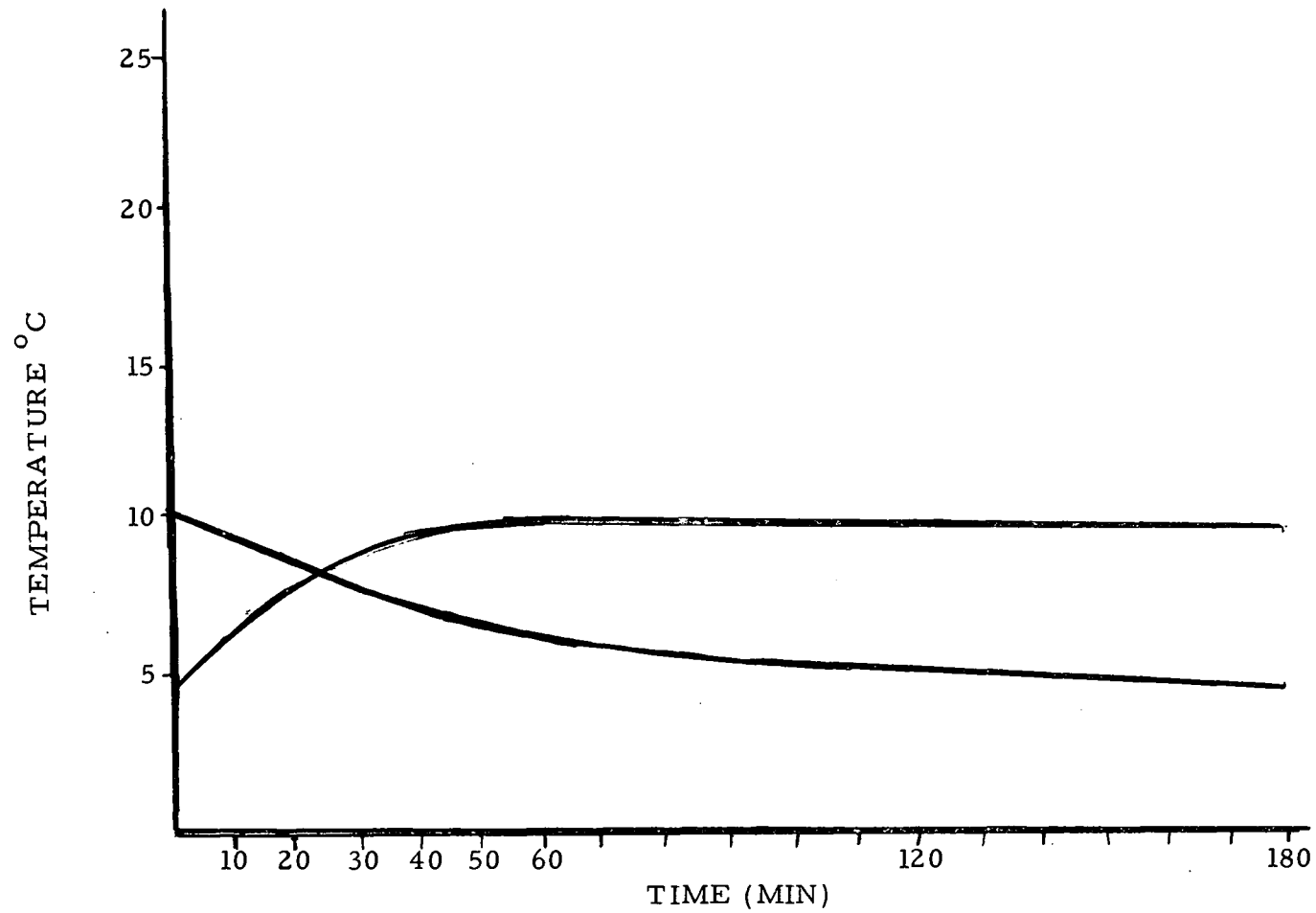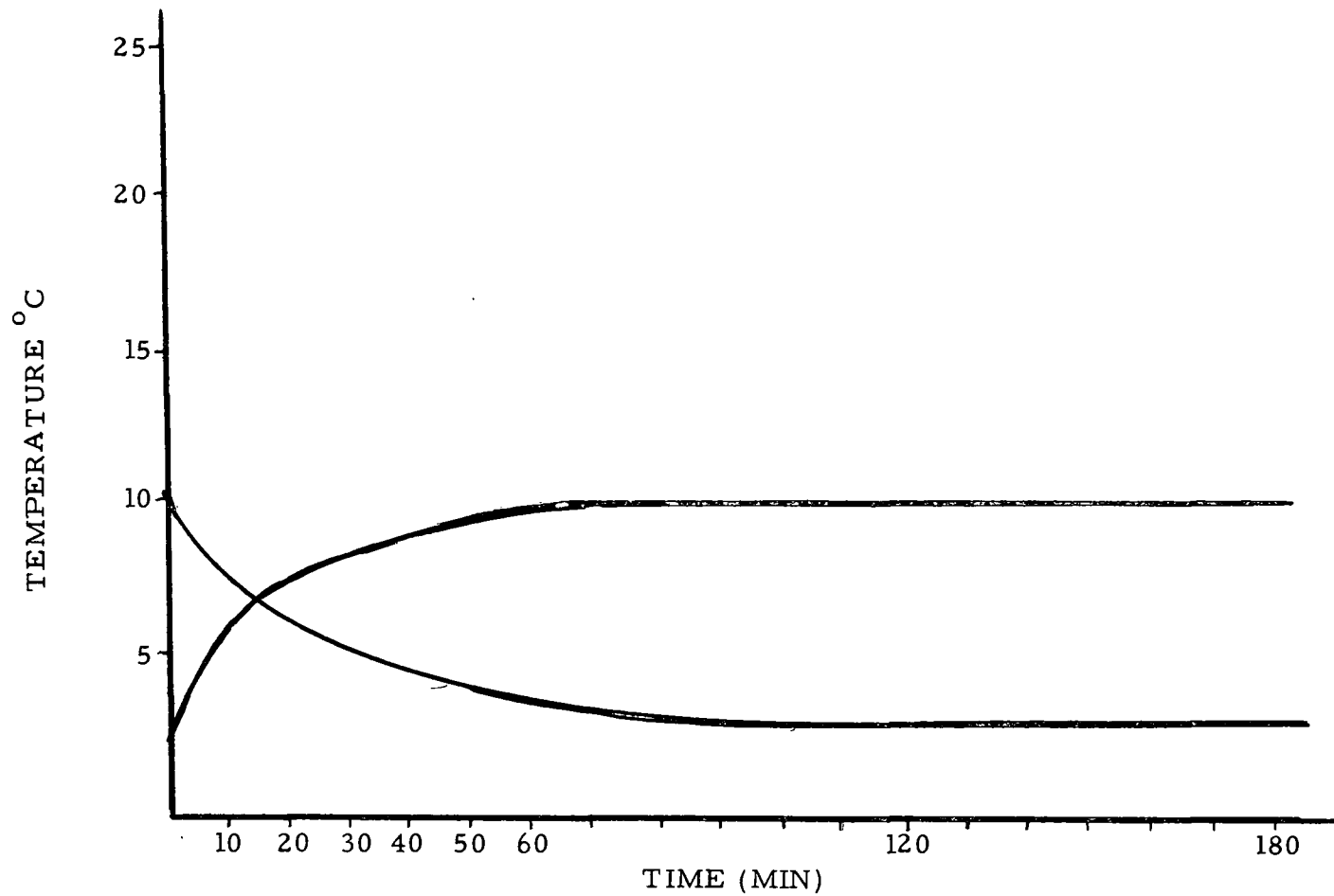Figure 4. Temperature Change Rate in Incubator Under Program II C (27°C-4.5°C)
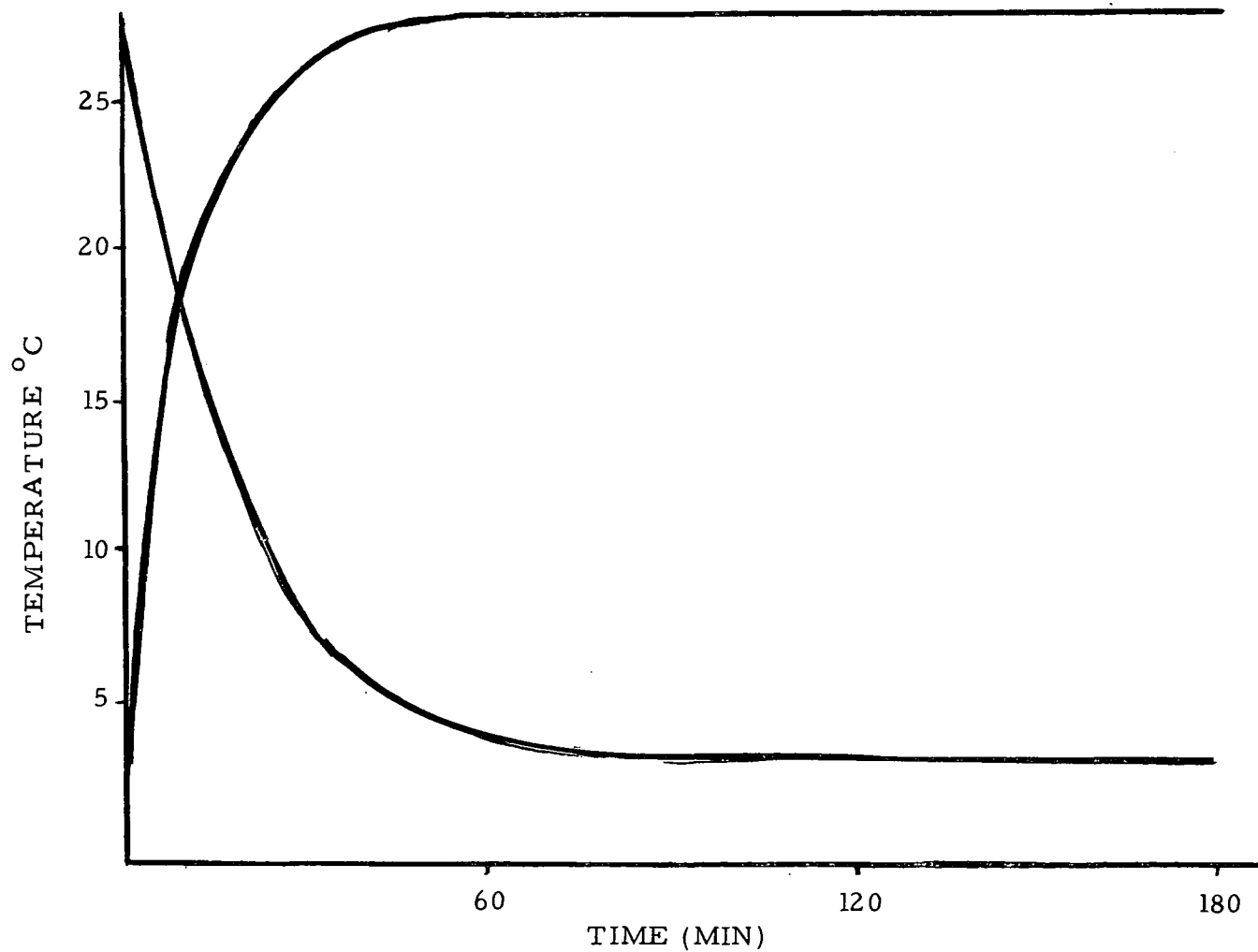
Figure 5.   Temperature Change Rate in Incubator Under Program II D (27°C-1.5°C)
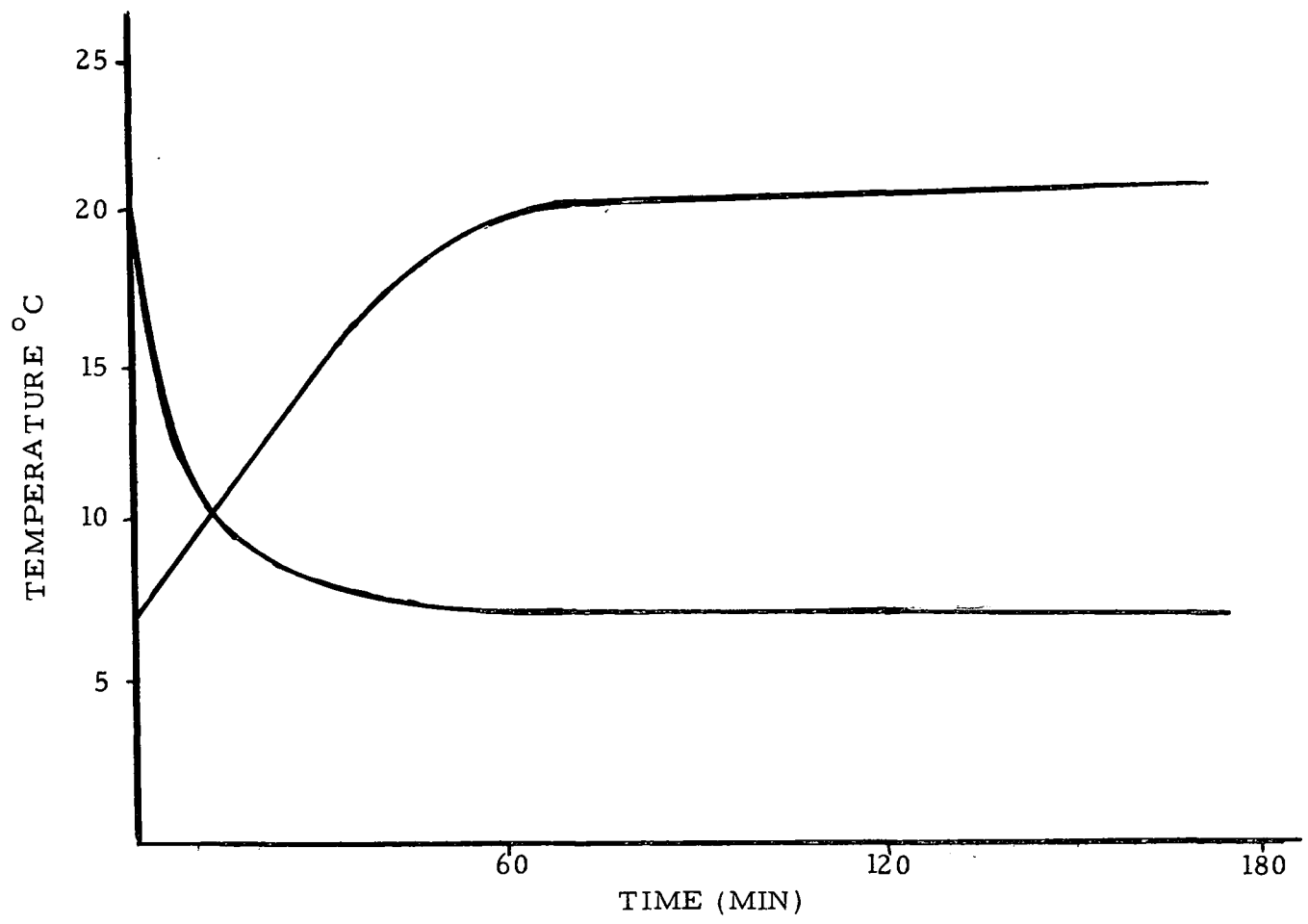
Figure 6. Temperature Change Rate in Incubator Under Program III B (21°C-7.0^{o}C)
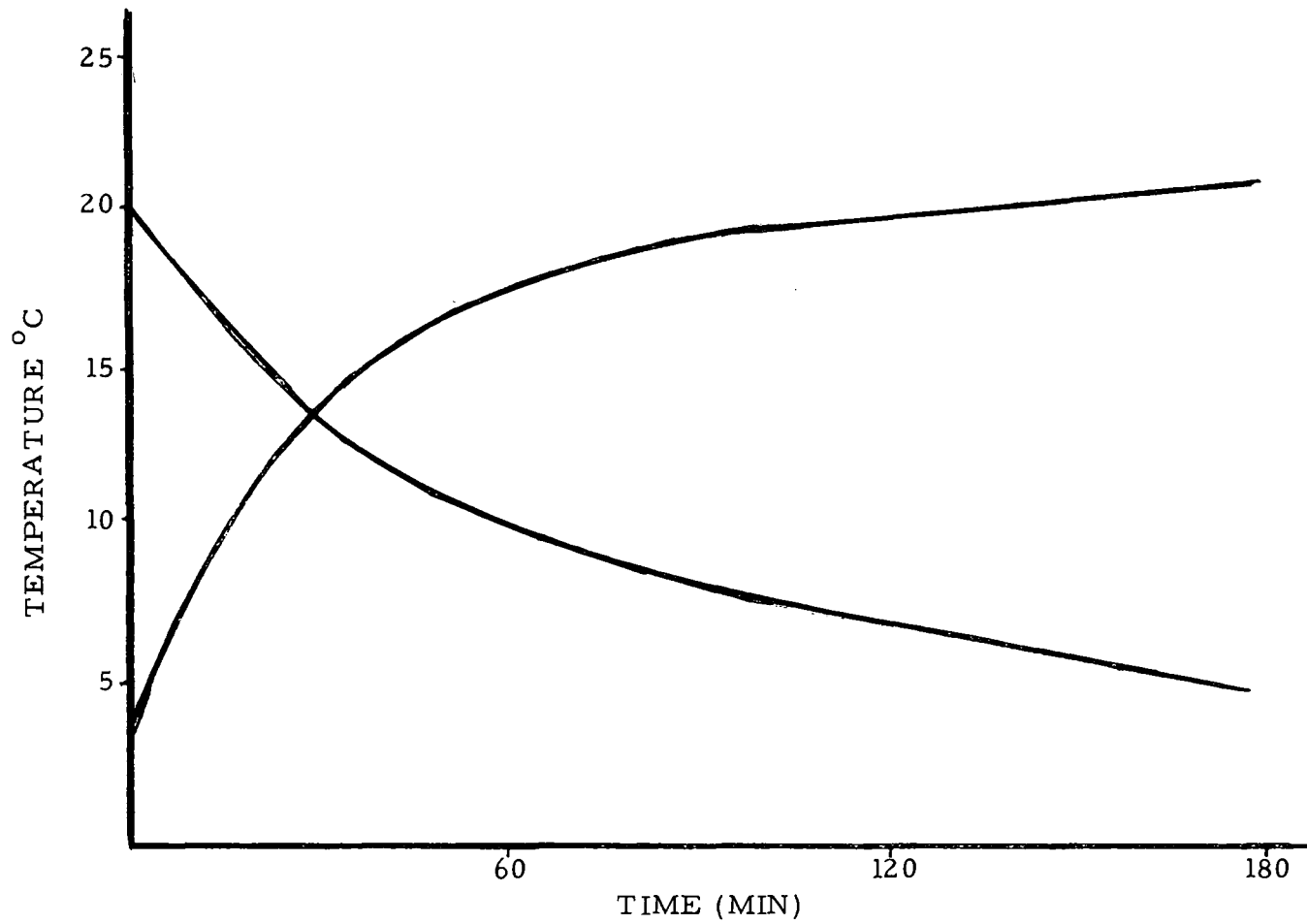
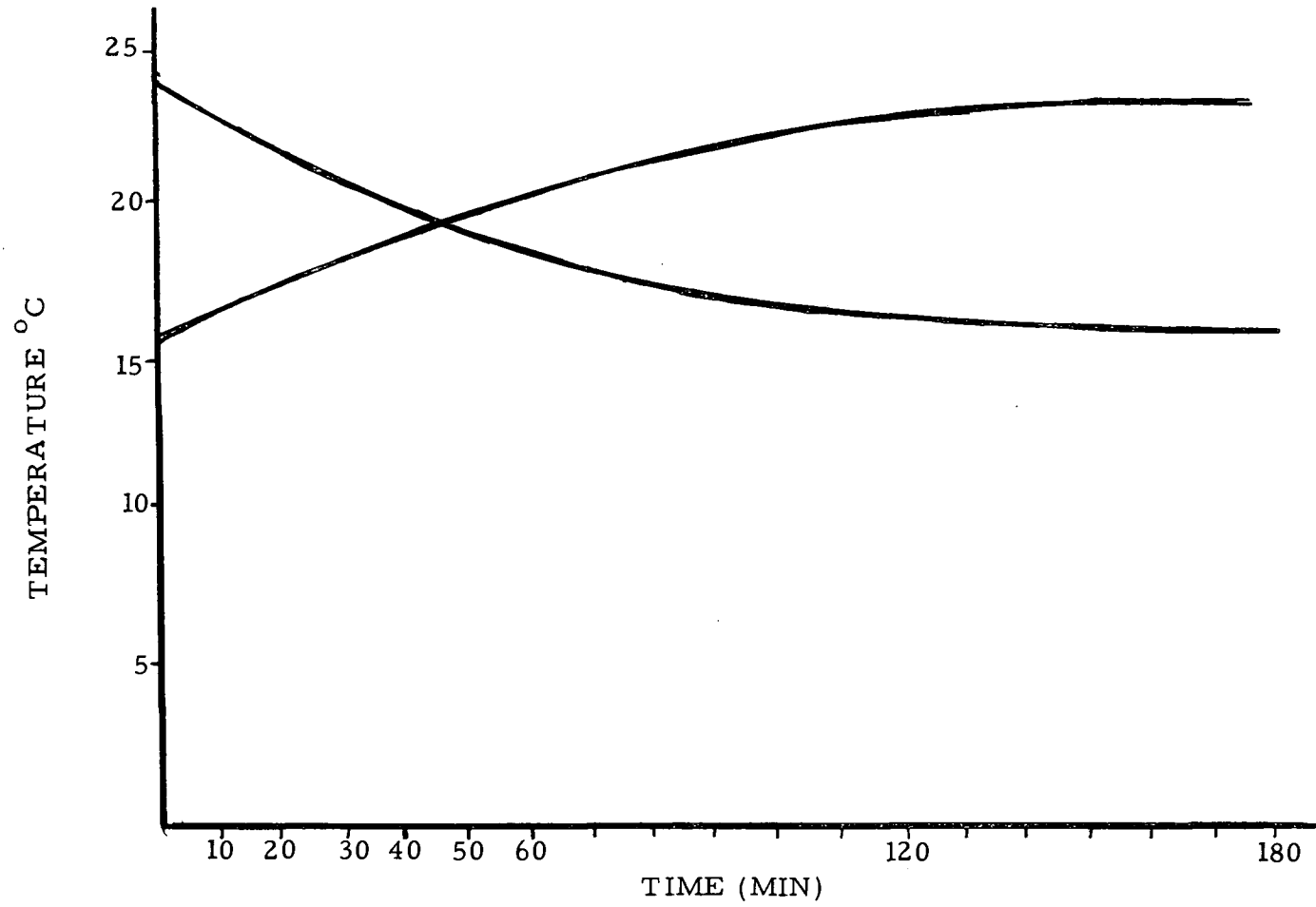Figure 7. Temperature Change Rate in Incubator Under Program III C (21°C - 4.5°C)

Figure 8. Temperature Change Rate in Incubator Under Program III D (21°C-1.5°C)

Figure 9. Temperature Change Rate in Incubator Under Program IV B (15.5°C-7.0°C)

Figure 10. Temperature Change Rate in Incubator Under Program IV C (15.5°C-4.5°C)

Figure 11. Temperature Change Rate in Incubator Under Program IV D (15.5°C-1.5°C)

Figure 12. Temperature Change Rate in Incubator Under Program V B (10°C-7.0°C)

Figure 13. Temperature Change Rate in Incubator Under Program V C (10°C-4.5°C)

Figure 14. Temperature Change Rate in Incubator Under Program V D (10°C-1.5°C)

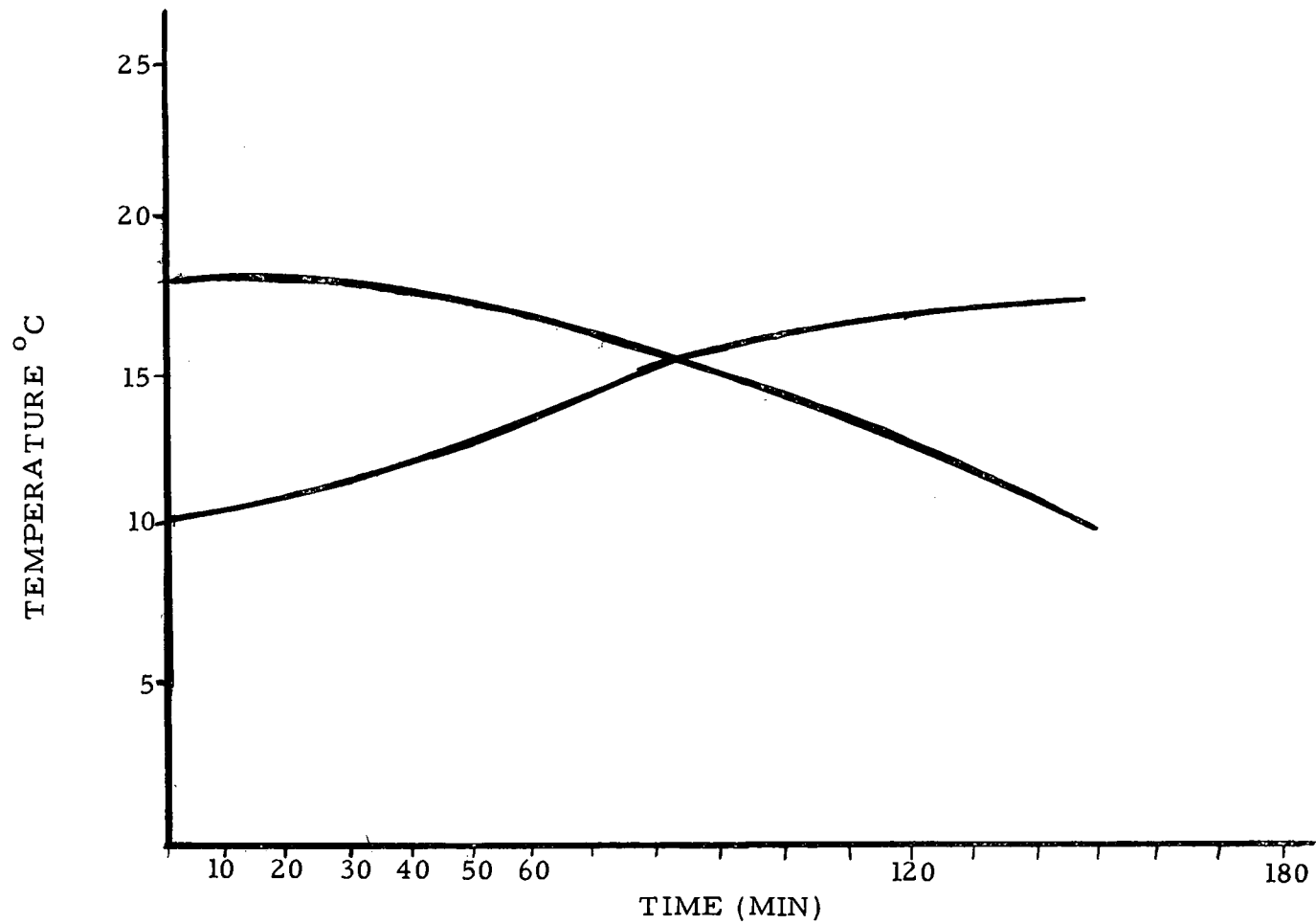Figure 15. Temperature Change Rate in Incubator Under Progam VIII (24°C-15.5°C)

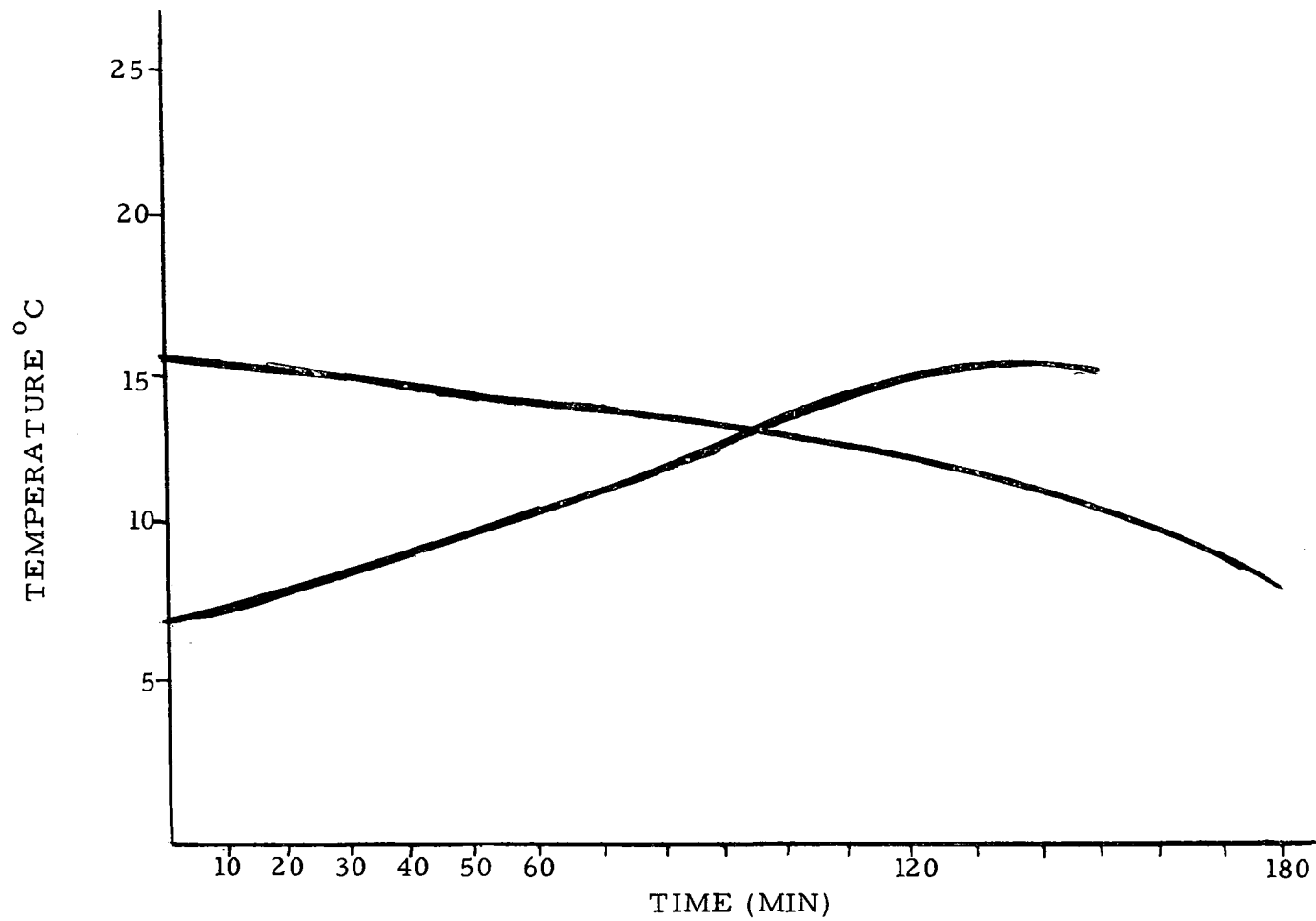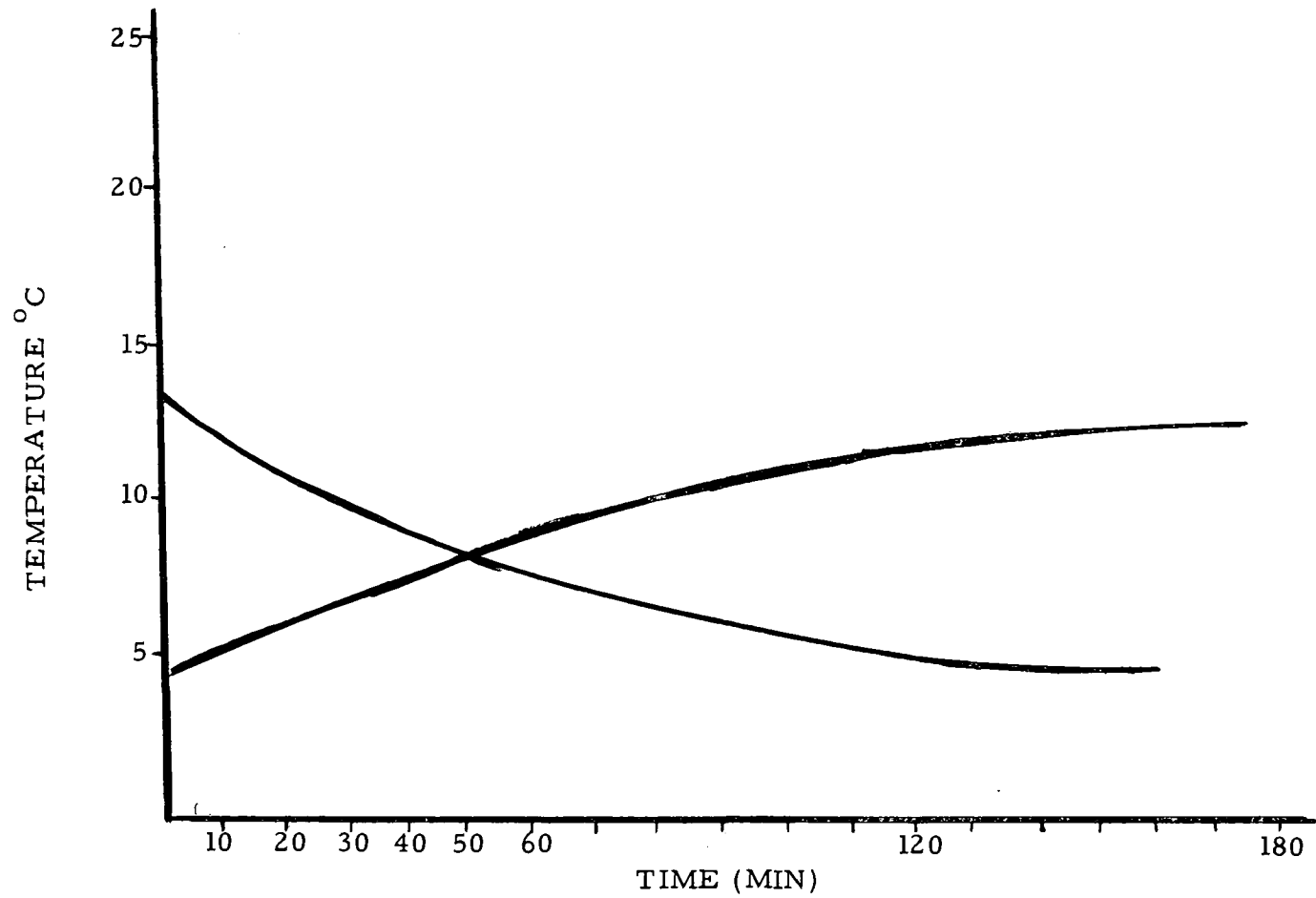Figure 16. Temperature Change Rate in Growth Chamber Under Program IX ($18^{\circ}C$-$10^{\circ}C$)

Figure 17. Temperature Change Rate in Growth Chamber Under Program X (15.5°C-7.0°C)

Figure 18. Temperature Change Rate in Growth Chamber Under Program XI (13°C-4.5°C)