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Temperature Change for Use in Cold Tolerance Tests of
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An apparatus was designed to permit freezing tests of needles and other tissue parts from coniferous trees under controlled rates of temperature change with time. The design is based on the principle of a balanced system of refrigeration demand and heat input to achieve a steady temperature state. The apparatus permits the operator to decrease temperatures from 0°C to -40°C at rates of 1, 2, 3, or 4°C per hour, to hold temperatures at the desired freezing level from 1 to 24 hours, and to increase to 0°C at rates of 1, 2, 3, or 4°C per hour. Temperatures achieved within the sample tubes are accurate to within $\pm 0.9^{\circ}\text{C}$ of the expected temperature.

Design of an Apparatus
with Controlled Rates of Temperature
Change for Use in Cold Tolerance
Tests of Conifers

by

William James Wessell

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DESIGN OF AN APPARATUS WITH CONTROLLED RATES OF TEMPERATURE CHANGE FOR USE IN COLD TOLERANCE TESTS OF CONIFERS

INTRODUCTION

Frost may kill or damage trees. Degree of damage is greatly influenced by their ability to withstand low temperatures. This ability, termed cold tolerance, is determined by a complex of ecological, physiological and genetic factors, and does vary between and within species. Knowledge of cold tolerance of forest trees is essential to good silvicultural practices. Information concerning cold tolerance may be obtained through observations in the field or through experimentation in the laboratory. The principal approach to tests of cold tolerance in the laboratory is artificial freezing of plants or of some of their tissue. This work was undertaken to provide an apparatus with which accurate tests of cold tolerance could be performed.

THE TOLERANCE OF COLD

The Freezing Process

The ability of a plant, or its various parts, to endure low temperatures has been variously named. "Cold tolerance" is used here to refer to that ability of a plant to tolerate temperatures which freeze its water, thereby removing that water from solution, and introducing inert foreign bodies, or ice crystals, into the biological system. The results (injury, death or otherwise) are direct or indirect consequences of this freezing of water (Meryman, 1956).

The freezing point of a plant's water is something lower than 0°C , that water being, to some degree, less than pure. The freezing point is rarely lower than -2.5°C in non-halophytes (Levitt, 1956). In most plants, supercooling is usually only a very few degrees, and the supercooled state is not of consequential duration (Levitt, 1966). When the freezing process does occur, it is subject to a complex of factors. Olien (1967) describes two freezing patterns of primary concern. The nonequilibrium freezing process is so named due to a sudden decrease in liquid, with the amount of ice formed not a continuous function of temperature. Ice crystals grow explosively at a uniform freezing temper-

ature. Their formation is favored by the presence of excess water, which is not closely associated with cell walls and protoplasts. By contrast, the equilibrium freezing process involves the continuous exponential decrease in non-excessive water as a function of temperature. Of this closely associated water, the least associated (with a higher freezing point) freezes first, at moderate freezing rates, in the spaces between protoplasts. The ice so formed has a vapor pressure lower than that of adjacent liquid, and water moves from protoplasts, through the plasma membrane to nucleation sites, thus desiccating the protoplast and contracting the cell. The ice crystals formed in biological systems are more or less complete hexagonal lattices. Complete lattices are formed by very slow freezing. Dendrites are formed by very rapid freezing. Slow freezing promotes ice formation external to the protoplast; very rapid freezing promotes internal ice formation.

The amount of water frozen is a function of temperature. Meryman (1956), working with animal tissues, reports a rapid increase in the production of injury from 0°C to -10°C . At this point, greater than 90 percent of the cell's water is frozen, and beyond this point, there is no increased dehydration of the cell, only chemical denaturation at an exponentially decreasing rate.

Meryman (1957) reports increasing solute concentration due to dehydration as temperature decreases from -10°C to -15°C . Beyond this point there is no significant increase in concentration. Levitt (1956, 1957) reports ice crystallization and attendant dehydration to at least -30°C , at which point 5 to 10 percent of the plant's water can remain unfrozen. Sakai (1966) considers it a reasonable assumption that extracellular freezing withdraws almost all freezable water from the cell at low temperatures near -20°C .

Sites of Ice Formation

The freezing process produces either intracellular or extracellular ice. Intracellular ice formation is favored by excessive water and rapid freezing rates. It is always fatal (Siminovitch and Briggs, 1953; Levitt, 1956). When tissues are sufficiently dehydrated, as by slow prefreezing to below -20°C , the protoplast can survive super-low temperatures (for example, -189°C). Prevention of ice nucleation within the protoplast by dehydration of most freezable water is essential to survival (Sakai, 1966). All protoplasm can survive these conditions. Only protoplasm which is not dehydrated is injured by low temperatures. In dehydrated protoplasm, no crystallization occurs (Levitt, 1956).

The important survival factor is the way in which the tissue

passes through the critical zone between 0°C and the "vitrification" temperature (Levitt, 1966). This temperature is in the range of -100°C to -130°C for the biological systems (Meryman, 1956). Freezing to below this temperature will or will not produce injury, depending on the degree of hydration of the tissue and the rates at which the tissue passes through the zone. In the critical zone, crystals are not stable, and recrystallization can occur upon freezing and thawing (Meryman, 1956). This is the basis for the injury due to slow thawing, which allows the probable growth of crystals within the cell which nucleated during the freezing to super-low temperatures (Sakai, 1966).

Extracellular ice formation occurs when the temperature drops below the freezing point at a slow rate. It may or may not injure the cell (Levitt, 1956). Protoplasts are adapted to undercooling due to the presence of the surrounding plasma membrane. The water in intercellular spaces is not adapted to undercooling. After a relatively slight undercooling, ice crystals form in intercellular spaces at slow freezing rates. The plasma membrane prevents these crystals from seeding into the protoplast. At this point, there is a vapor pressure difference between the extracellular ice (lower pressure) and the protoplast. Available water diffuses from the protoplast, through the plasma membrane, and freezes on the growing ice mass. Protoplasmic concentration of solutes increases and cells contract. The freezing point of the protoplast decreases, and the freezing boundary is maintained

outside of the cell (Meryman, 1956; Levitt, 1956). Extracellular freezing occurs as an equilibrium freezing process, although nonequilibrium freezing can occur following supercooling or when there is excessive water in the intercellular spaces (Olien, 1967).

Freezing Injury

Knowledge of the mechanism of cold injury is uncertain. General agreement exists that the formation of ice is the direct or indirect cause of injury (Meryman, 1956; Levitt, 1956, 1966; Parker, 1963). At sub-freezing temperatures, the majority of plants cannot avoid ice formation. Lack of injury is generally not due to frost avoidance, but to frost tolerance (Levitt, 1966). If it occurs, the injury produced by extracellular ice is quantitatively related to the amount of ice formed. However, injury is caused not by the amount of ice formed, but by the amount of water removed from the protoplast by frost dehydration (Levitt, 1956). The relationship between dehydration and frost injury is supported by the positive correlations between injury due to dehydration by plasmolysis and desiccation and injury due to extracellular freezing found by Siminovitch and Briggs (1953).

Dehydration of the cell by extracellular ice causes both the cell wall and the protoplast to contract, with ice crystallizing in the intercellular spaces. This differs from true plasmolysis, in which only the protoplast

contracts, receding from the cell wall (Siminovitch and Briggs, 1953). Where frost injury does occur, the cell wall expands upon thawing, but the protoplast remains contracted. This is due to the inability of the dead protoplast to reabsorb the water. This phenomenon is known as "frost plasmolysis." Uninjured cells reabsorb the water and return to a normal appearance. Injured cells always lose their semipermeability (Levitt, 1956).

The dehydration of the protoplasm causes its increased consistency, or stiffening, and the increased concentration of the cell's contents (Levitt, 1956; Meryman, 1956). The dehydration is apparently limited, not by the zero water content of the cell, but by the degree of binding of the water in the cell (by proteins, sugars and other molecules), which at some point prevents further dehydration (Meryman, 1957). Further dehydration may be prevented by a vapor pressure difference, between internal liquid and external ice, which provides insufficient energy for dehydration (Olien, 1967).

The exact cause or causes of injury are obscure. Two major schools of thought exist concerning causes of injury. The older of the two considers damage to be the consequence of a physical process. According to this view (Levitt, 1956, 1966; Parker, 1963; Olien, 1967) injury occurs in frost-dehydrated protoplasm due to mechanical damage caused by tensions resulting from both increased protoplasmic consistency and cell contraction.

The damage can occur during freezing, while frozen, or during thawing of the cell. Injury due to intracellular ice is easily explained by mechanical disruption of the protoplast. Newer theories postulate that the causes of injury are of biochemical nature (Meryman, 1956, 1957). Perhaps the best known of these is the sulfhydryl-disulfide theory (Levitt, 1966).

According to this theory, injury results from the unfolding and mutual approach of protoplasmic protein molecules due to dehydration by freezing. Denaturation occurs following formation of intermolecular disulfide bonds by sulfhydryl groups.

Cold Tolerance

Just as the exact nature of freezing injury is unknown, the method or methods by which plants tolerate freezing are unknown. The more likely theories are related to dehydration and protoplasmic properties. The tolerance of plants to freezing is dependent upon freezing conditions as well as physiological factors (Olien, 1967). Levitt (1956) specifies standardized conditions for testing frost tolerance because hardiness is not fixed in a plant of the same level all the time.

Succulent and tender tissues are generally killed at or very near their freezing point, and freezing or thawing rates make little difference (Levitt, 1956). In these tissues, the nonequilibrium freezing process occurs, destroying the protoplasts (Olien, 1967). Injury readily results from ice

formation, and these plants are not said to be frost tolerant. The transition between the stages of tenderness and hardness is marked by the ease of intracellular ice formation. For the hardened plant, rapid rates of freezing are required for the fatal intracellular ice formation. Moderate rates produce only extracellular freezing (Olien, 1967). When the rate of freezing is sufficiently rapid, either excessive supercooling of all cell fluids eventually favors intracellular ice, or the diffusion of water from cells to existent sites of external ice is insufficient to prevent intracellular ice formation (Siminovitch and Briggs, 1953). The faster diffusion rates of hardy cells are related to the permeability of the cell membranes, and these are more permeable to water and polar solutes in hardy cells (Levitt, 1956).

Levitt (1956, 1966) also reports that hardening cells in trees and some herbaceous plants have increasing cell sap concentrations due to sugar accumulation from starch conversion. The results are more solutes, therefore osmotic binding of water and crytoidally bound water increases. This increased concentration of cell sap is neither strictly correlated with hardness, nor is it a sufficient explanation for all increases in frost hardness. The starch-to-sugar conversion is apparently controlled by both temperature and photoperiod. Colder temperatures favor sugars. Shorter photoperiods may trigger the activation or accumulation of certain enzymes. The effect of increased vacuolar osmotic potential, due to increased solute concentration, is decreased hydration of the protoplasm. This is thought to produce a shift

in the hydrophily of protoplasmic proteins to oppose dehydration, resulting in a greater protoplasmic binding of water. In less hardy plants, dehydration decreases hydrophily, through its effect on proteins, to a greater degree than in more hardy plants. When subject to frost dehydration, the hardy plants can withstand dehydration stresses to a greater degree, their protoplasm being more hydrophilic than that of less hardy plants. Their protoplasm is also more ductile, or less stiffened.

Whatever the exact nature of the cold tolerance mechanism may be, plants do exhibit seasonal variation in cold tolerance, in apparent response to environmental factors. Levitt (1966) supports the general theory that those factors conducive to growth and development do not favor hardiness. The converse is also theorized. Although hardiness and dormancy are moderately correlated, Irving and Lanphear (1967) demonstrate hardening independent of bud dormancy. Hardiness was shown to increase with an increasing number of short days followed by low temperature hardening at night. This photoperiodic response may support Levitt's (1956) suggestion of enzyme activation or accumulation due to a shorter photoperiod. The low temperature treatment (below 10°C, 0°C to 5°C is best) is likely effective through starch-to-sugar conversion. Sufficient illumination is also believed important for photosynthetic production of food required in the hardening process (Parker, 1963; Levitt, 1956, 1966). These environmental conditions vary seasonally to increase hardiness in late summer or early fall, and to

decrease it in late winter or early spring. This seasonal trend in hardiness has been repeatedly demonstrated.

Takatoi, Watanabe, and Kamada (1966) report differential seasonal hardiness between fir and spruce buds, and between twigs and buds, with a reversal of the latter difference during the winter. Parker (1955) found little interspecific difference in hardiness between excised leaves of grand fir and ponderosa pine, both species showing rapid increase in hardiness in early November to at least -55°C killing temperature in January, and a rapid decrease in hardiness in late winter. Parker (1957) reports a minimum killing temperature of -47°C for ponderosa pine twigs and leaves in November and March, with lower unattained killing temperatures through mid-winter. A marked increase in hardiness during September and October, and a marked decrease in spring were also reported. Working with eastern white pine, eastern hemlock, and mountain laurel, Parker (1961) found no interspecific differences in summer hardiness at -8°C , but a cooler September brought increased hardiness in pine, with the first autumn frost producing further hardiness differentials between the three species. Substantially warmer weather in spring produced rapid dehardening, again with species differences. Eguchi, et al. (1966) report seasonal variations, and differences between morphological groups, of hardiness in cryptomeria twigs. These and other examples should be considered only individually for, as will be discussed later, comparisons should not be made unless test conditions are similar.

Parker (1963) reviews cold resistance in woody plants. Seasonal, interspecific, intraspecific, tissue and organ, and test condition variations in hardiness are well documented in the literature.

TESTING COLD TOLERANCE

Methods

The most realistic appraisal of a plant's cold tolerance is by the field survival under conditions of critical stress. The utility of field survival as a measure is limited by the infrequent occurrence of severe winters, which occur on an average of once in ten years (Levitt, 1966). More frequent occurrence may be found under special conditions. The frost damaged plantation is one example. Johansson and Torssell (1956) employed a portable freezing apparatus for artificial trials of crop plants in the field. The inconvenience of physical limitations and the complications of uncontrollable environmental conditions lessen the utility of such apparatus.

Better control of conditions is achieved by the use of low temperature chambers for growing and hardening plants, as well as for actual freezing trials (Dexter, 1956). In this way, actual cold tolerance tests may be performed in the laboratory, with the frequency and control not possible in the field. Dexter (1956) and Levitt (1966) report good to excellent agreement between artificial freezing tests and actual field survival.

Among other tests for cold tolerance, the method of dehydration by desiccation and plasmolysis produces comparative hardness values as dependable as those produced by actual extracellular freezing tests (Siminovitch and Briggs, 1953). Levitt (1966) is of the opinion that the

deplasmolysis method is the only workable test of tolerance other than actual freezing tests. Other tests, for example those based on electrical properties of tissues and cell contents, respiration and cell sap properties, as well as dehydration tests, ultimately must derive their potential validity from correlations with actual freezing tests. Each of these and other tests measures factors possibly associated with the tolerance mechanism. In view of the elusive nature of the tolerance mechanism, the need for actual freezing tests is apparent.

Freezing Tests

In comparative studies of cold tolerance using actual freezing tests, it is essential that close control be exercised over standardized conditions of freezing and thawing rates, time frozen, and post-thawing conditions. Freezing rate can influence the location (intercellular or extracellular) of ice, particularly in less hardy tissue. Where extracellular ice occurs, if injury is not irreversible upon freezing, it is contingent upon post-freezing factors. Once equilibrium is reached and undercooling is avoided, one or several hours frozen have little effect on injury, unless the time frozen is more than one day. Fast thawing rates may result in injury not otherwise occurring, due to possible inability of the protoplast to take up water at the rate of thawing. Unfavorable post-thawing conditions, for example high temperatures, may result in injury not otherwise occurring. The requirements for

control over these conditions are based on the possibility that between no injury and irreversible injury, extracellular ice formation may produce potentially injurious conditions which will be effective if these factors are unfavorable (Levitt, 1956).

The selection of freezing rates for practical tests should be made to favor extracellular ice formation. Tender tissues are susceptible to intracellular ice at relatively slow rates. Although conditions favoring intracellular ice in more hardy tissue may occur in nature (Levitt, 1966; White and Weiser, 1964), extracellular ice is considered the predominant occurrence (Siminovitch and Briggs, 1953; Levitt, 1956; Salt and Kaku, 1967). Levitt (1966) defines slow freezing for whole plants, and presumably for intact leaves and other plant parts, as 1°C to 2°C per hour, and fast freezing as 5°C to 20°C per hour. Parker (1955) used a "slow" freezing rate of 1°C per hour, and a thawing rate of about 1.5°C per hour for conifer leaves. He considered it apparent that sudden changes in temperature are more damaging than simply low temperatures. His later work (1959, 1961) reflects this possibility, with freezing rates of 3°C and 4°C per hour, and thawing rates of 4°C and 8°C per hour, for similar material. Midwinter resistance to injury at -80°C in slowly frozen conifer leaves (Parker, 1961) indicates possibly total tolerance under more practical conditions.

TESTING APPARATUS

Types of Apparatus

Basic components of testing apparatus are a freezing chamber, refrigerant, cooling medium, and a temperature measurement system. Apparatus commonly used in practical studies with woody species show much variation in principle and performance. Glerum, Farrar, and McLure (1966) used an insulated fibre drum with a dry ice add-subtract method of temperature control. Irving and Lanphear (1967) used a stepwise freezing procedure. Styro-foam sample containers were transferred to freezers at successively decreasing temperatures in steps of 5°C to 6°C. Temperatures in containers were measured at 2 1/2-minute intervals. Thawing was in plastic containers at room temperature and high humidity. Eguchi, et al. (1966) also used a successive freezing temperature procedure, with 2 1/2°C and 5°C steps. Thawing was in air at 0°C. White and Weiser (1964) placed flasks with samples in a freezer and lowered temperature at an approximate rate, then removed flasks at successive temperatures of decreasing values. The flasks were thawed in progressively increasing steps in cold chambers. Siminovitch, et al. (1964) used a commercial apparatus with a manual temperature adjustment and an accuracy of 0.84°C for freezing, while thawing in air at +3°C for 18 to 24 hours. Salt and Kaku (1967) used a thermoelectric cooler and two concentric glass vials to slow the freezing rate to 1°C to 3°C per minute.

Havis (1964) used an agitated methanol bath with samples contained in tubes. This bath provided temperature control within 0.56°C . Siminovitch and Briggs (1953) used a commercial apparatus with a dry ice refrigerant, from which a blower passed cold air through a manually dampened duct to a test chamber, in which a heater was located. The heater and blower were activated separately or together through manual control of a thermostat and relay. Control of temperatures from 0°C to -70°C was achieved within $\pm 1^{\circ}\text{C}$. The temperature was decreased by manually setting the regulator to successive temperatures in 1°C steps. Thawing was at -1°C or in water at room temperature. Greenham and Daday (1960) also used an air-medium chamber, with blown air cooled over refrigerator coils and heated by the electric fan motor. A second fan provided mixing of the air. Control was achieved by a 24-hour cam which regulated the position of one of a pair of contact points. The other contact point followed the rotary motion of a thermostat stem. When the contacts were open the refrigerator operated, decreasing the temperature and turning the thermostat contact toward the cam-controlled contact, shutting the refrigerator off upon contact. The cam was programmed for a holding period, decreasing temperature period, holding period, increasing temperature period, then holding period at starting temperature. This gave a standard program for comparative trials. Parker (1955) used a refrigerated chamber, into which he placed flasks containing ethylene glycol and samples in six test tubes. After preliminary low temper-

ature equilibrium was reached, the refrigerator thermostat was set to a lower temperature in successive steps when the preceding temperature was reached in the test tubes. Thawing was by warming a flask with contents to room temperature, giving a thawing rate which was "faster at first."

Initial Apparatus

The development of this temperature control apparatus began with a perviously built freezing chamber, consisting of a thermostatically controlled 1/3-horsepower motor and compressor using Freon 22, and a 12" diameter, 12" deep copper drum centered within a 3'x3'x3' 3/4" plywood cabinet filled with styrofoam insulation (Figure 1). The refrigerator coils encircle the copper drum. The original cooling medium was glycerin, which was extremely viscous at the minimum attainable temperature near -50°C . Preliminary measurements revealed steep radial temperature gradients within the glycerin bath. The cooling rate was not linear.

Successive manual settings of the thermostat do not give a linear rate. They only segment the inherent curve, and are likely to introduce a gross stepping effect. An attempt was made to linearize the curve by interrupting the refrigerator circuit, at intervals of a few minutes, by means of a revolving table with a "sawtooth" cam actuating a microswitch. The grossly empirical nature of the method made it unacceptable.



Figure 1. The complete freezing apparatus.

Requirements

It was decided that accurate tests of cold tolerance must begin with a satisfactory apparatus, one designed to provide reproducible, controlled temperature conditions determined by the nature of the research. The apparatus is intended to be used as a means of determining and following the seasonal variation in cold tolerance of various tissues of interspecific and intraspecific plant groups, under freezing conditions most likely found in nature. This consideration, in view of the freezing process and its effects on ice formation, led to the selection of freezing and thawing rates of 1, 2, 3, and 4°C per hour. These rates must be uniform throughout the freezing and thawing periods, not "faster at first." It is desirable to select various lengths for the period during which the frozen state is held. Minimal temperature variation between samples during each trial is essential. Finally, a minimum of attention to the apparatus during trials was found to be convenient.

Recorder

The apparatus includes a Brown Electronik strip chart recorder with a 0°C to -50°C range. A 24-gauge copper-constantan thermocouple with a fused junction is used as the temperature sensor. The junction is located in air inside one of 24 test tubes, and the wire passes through a rubber stopper. From this point it is shielded over its six-foot

length to the recorder. Chart speed is 30 inches per hour. The chart paper is printed for 0°C to $+50^{\circ}\text{C}$, but 0°C is read as -50°C , and $+50^{\circ}\text{C}$ is read as 0°C . The millivolt span for each range was accounted for graphically to check validity of interchanging paper. Negligible differences were found across the temperature scale. Calibration of the recorder and thermocouple, using freezing mercury (freezing point -38.9°C) and salt solutions (freezing points -1°C , -3°C , and -5°C) showed accuracy to be $\pm 0.3^{\circ}\text{C}$. The same accuracy was obtained using the complete apparatus system.

Stirring Assembly and Bath

The viscosity problem of glycerin was avoided by substitution of a 60 percent ethylene glycol solution (freezing point near -50°C). The cooling curve for 18 liters of this solution, with refrigerator on constant demand, is shown in Figure 2A. The "free" warming curve is shown in Figure 2B. The minimum cooling rate for the 18-liter solution was 3°C per hour in the -45°C range. Since the maximum desired freezing rate is 4°C per hour, this rate must be attained at the lowest operating range. This was accomplished by reduction of the bath volume to nine liters. The cooling rates obtained were a maximum 15°C per hour in the -5°C range, and 4°C per hour in the -45°C range.

A uniform bath temperature, as sensed in the test tubes, was achieved by design of a stirring assembly (Figure 3 and Figure 4). This assembly is

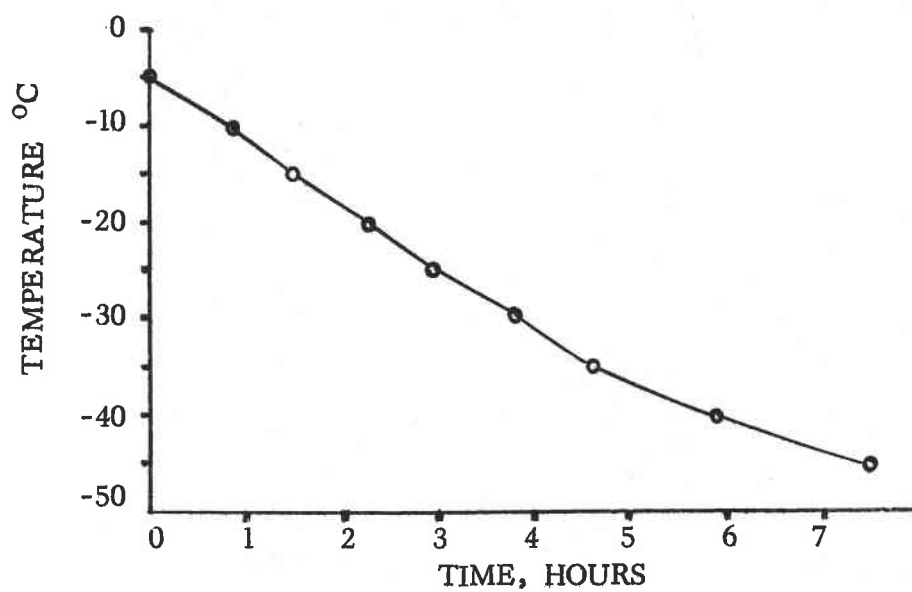


Figure 2A. Representative cooling curve for bath

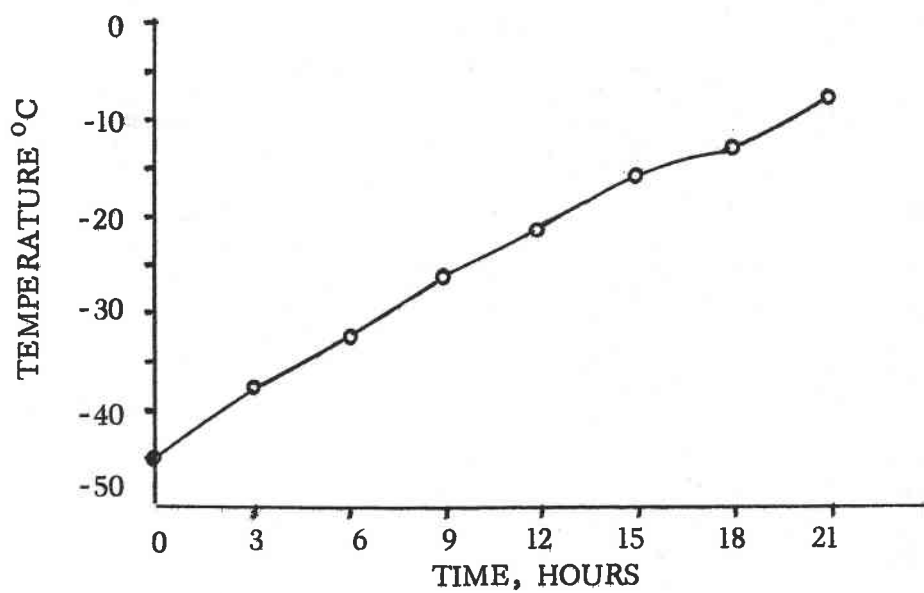


Figure 2B. Representative warming curve for bath



Figure 3. The stirring assembly

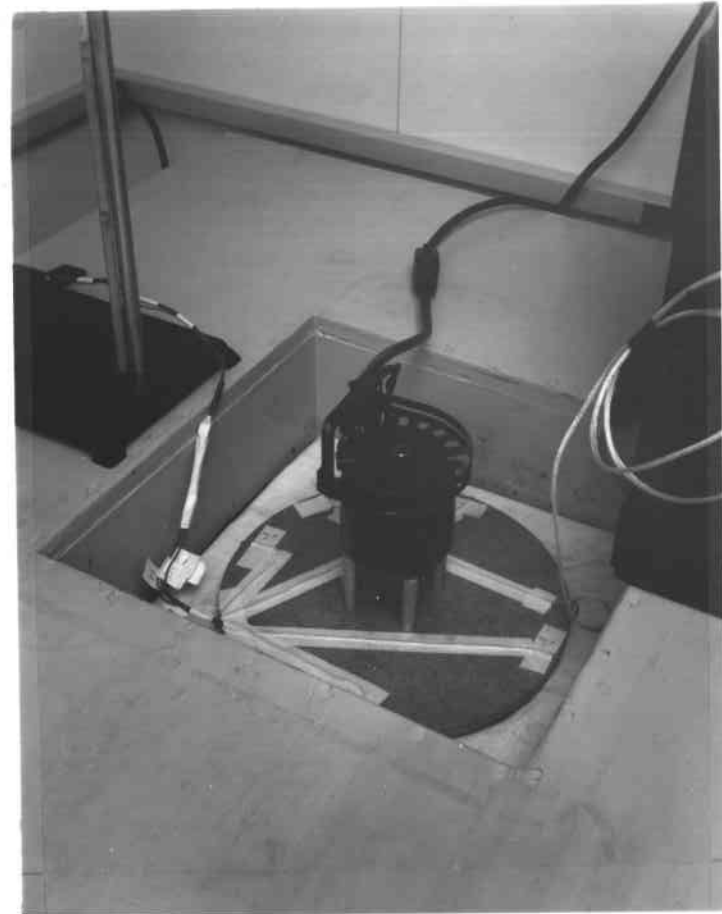


Figure 4. The stirring assembly in place

mounted on the circular center section of a four-inch thick insulating cinder block, which fits the 14"x14" chamber well. The weight of the assembly on its tapered sides provides a sufficient seal between the tapered faces. A small electric motor is mounted on the composition board top of the assembly. A 1/4" extension shaft is sleeve-fitted to the motor. It extends through a 3/8" PVC pipe, reaching to the bottom of the drum. At this point, a 3 1/4" blade is bolted to the shaft. The blade circulates the solution downward over the walls of the drum (the system boundary) and upward onto and around the 24 25x100 mm. Pyrex test tubes for tissue samples. These tubes are sealed with rubber stoppers and placed in two concentric circles of 12 holes each in a 1/16" thick, 7 1/2" diameter aluminum tray. This tray also has an inner circle of smaller holes to allow upward circulation. A second aluminum disc with matching circulation holes serves to secure the tubes. It is bolted at two points to the tray. The tray assembly is threaded to the shaft housing. When in place, the entire tray assembly is immersed in the bath. The temperature variation between test tubes will be discussed later (see Results of Operation).

Control Apparatus

Regulation of the bath temperature, as sensed in an immersed test tube by the control thermocouple, is based on the principle that for a given point within the range of 0°C to -50°C, a balance between refrigeration demand and heat input will result in a steady-state within the defined system of the bath.

The heat content per degree Centigrade of this nine-liter solution was calculated to an approximate value of 7000 calories per degree Centigrade. ¹ Cooling at a maximum rate of 15°C per hour in the -5°C range, the refrigerator demands 29 calories per second. ² To balance this refrigeration demand, a maximum heat input equivalent to 122 watts is necessary. ³

An immersion type heating tape was selected for placement in the bath around the inside of the drum. A variable autotransformer controlled the output of this heating tape (Appendix II). Each degree Centigrade per hour is equivalent to 8.13 watts. ⁴ Therefore, from the maximum of 15°C per hour in the -5°C range, to the minimum of 4°C per hour in the -45°C range, the heating element output must vary from 122 watts to approximately 33 watts. ⁵ Expressed as voltages (based on tape resistance of 92 ohms), these values give a desired autotransformer output of 108 volts at -5°C and 54

¹For both ethylene glycol (1) and water (2) the following calculations were made, using approximate values:
 (Specific gravity)x(Density of water)x(Volume)x(Specific heat)=(Heat content /°C)

(1) (1.1155) (1000 gm/l) (5.5 l) (0.515 cal/gm/°C) = 3,140 cal/°C

(2) (1.000) (1000 gm/l) (3.5 l) (1.012 cal/gm/°C) = 3,540 cal/°C

Adding (1) and (2) = 6,680 cal/°C = 7,000 cal/°C approx.

²15°C/hour=0.00415°C/sec., (0.00415°C/sec.) (7,000 cal/°C)=29 cal/sec.

³1 cal. = 4.2 joules, 29 cal/sec = (29) (4.2) joules/sec=122 joules/sec=122 watts.

⁴Demand at 15°C/hour = 122 watts, (1/15°C/hour) (122 watts)=8.13 watts/°C/hr.

⁵(15-4) (°C/hr) = 11°C/hr, (11 °C/hr) (8.13 watts/°C/hr)=89 watts, approx.
 (122-89) watts = 33 watts.

volts at -45°C . ⁶ Figure 5 shows the refrigeration demand curve (expressed as rates, $^{\circ}\text{C}/\text{hour}$, determined by sampling segments of the cooling curve across the range) and the heater-output curve which results from matching the end-point voltages to the corresponding points on the temperature scale. ⁷ Fortunately these curves are closely matched. The correctness of the method was verified by a very satisfactory approximation of a steady temperature in the bath with the values shown.

The need for a physical means of correlating the heater/autotransformer and the refrigerator with the actual temperature of the bath was satisfied by the travel of the pen carriage of the recorder. This pen became the linear equivalent of a rotating thermostat stem. To it was attached a T-bracket with two trippers mounted on it (Figure 6A and Figure 6B). The position of the tripper, therefore the temperature, was bounded with two microswitches on a carriage, one controlling the refrigerator operation, the other controlling the heater operation. At a given scale position, a temperature drifting upward (to the right, facing the recorder) caused the tripper to open the heater circuit, and the refrigerator "pulls" the pen back to position. When

$$^6 P/R = I^2, 122/92 = 1.33 = I^2, I = 1.17 \text{ amps.}$$

$$33/92 = 0.35 = I^2, I = 0.59$$

$$E = IR, (1.17)(92) = 108 \text{ volts, } (0.59)(92) = 54 \text{ volts.}$$

⁷The -5°C to -45°C equivalent of 108 volts to 54 volts requires an arc of approximately 134° to be turned by the autotransformer brush giving a linear (volts/arc $^{\circ}$) output. The voltage output through the arc, projected to a straight line of corresponding degrees Centigrade, namely the temperature scale, results in the curve shown for the heater.

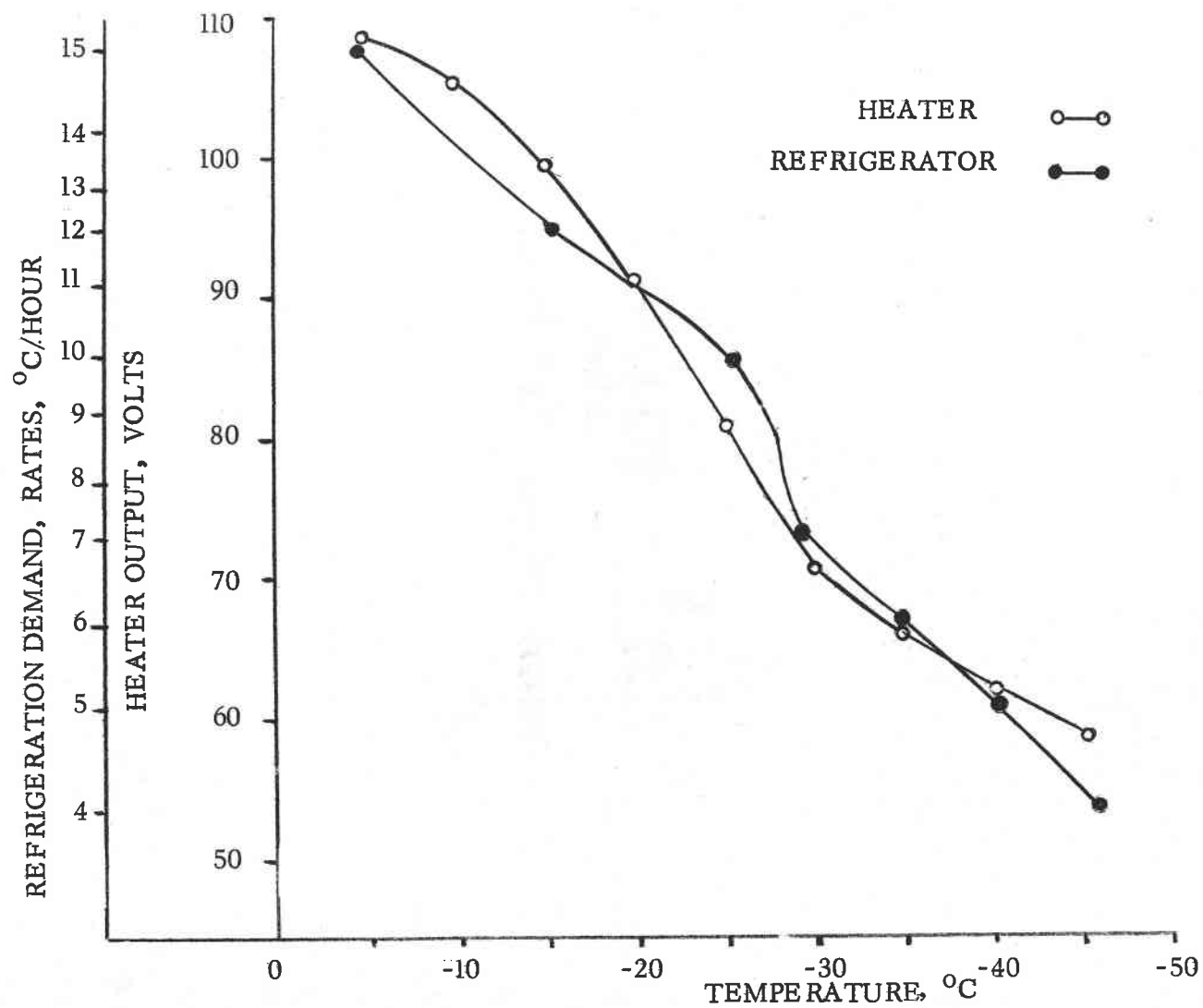


Figure 5. Matched refrigeration-demand and heater-output as a function of bath temperature

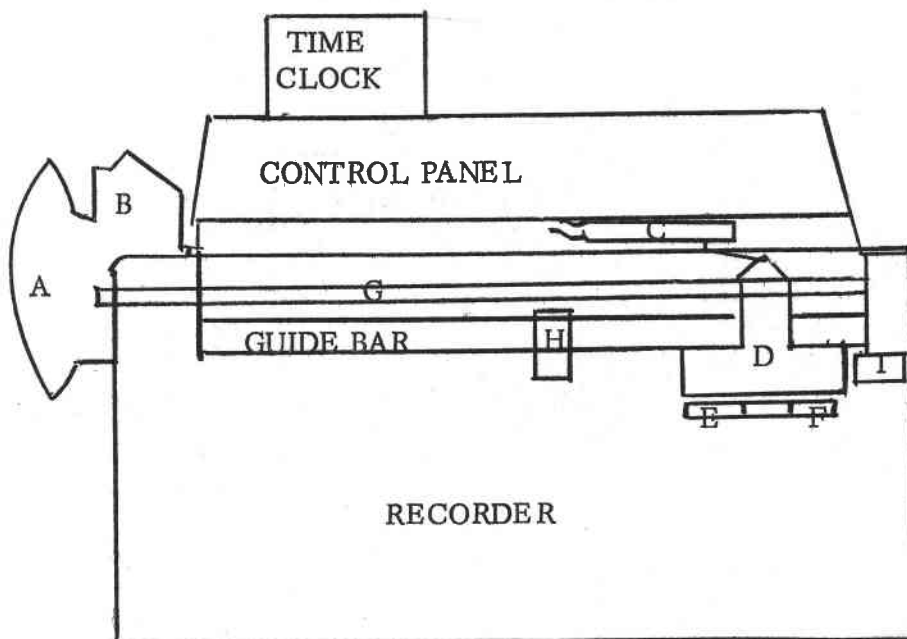
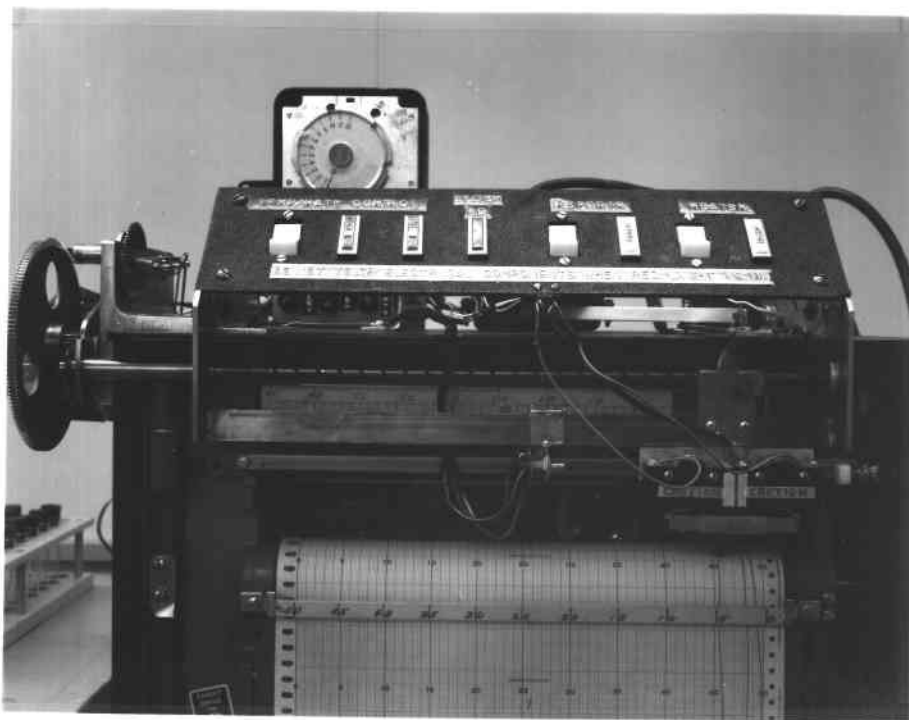


Figure 6A. The recorder and temperature control apparatus

Figure 6B. Diagram of the recorder and temperature control apparatus

- A. Rate control gear train
- B. Gear storage
- C. Slotted arm connecting autotransformer to micro-switch carriage assembly
- D. Microswitch carriage assembly
- E. Refrigerator microswitch tripper
- F. Heater microswitch tripper
- G. Microswitch carriage drive shaft
- H. Lower limit switch
- I. Upper limit switch

the pen crosses (right to left) the center point between the microswitches, the heater circuit closes and the refrigerator circuit opens. A similar operation occurs when temperature direction reverses. Any drift in temperature due to imperfect matching of continuously "on" cooling and heating systems is corrected by opening the circuit of the over-balancing element.

There is no physical connection between the trippers mounted on the pen carriage and the microswitch arms. The light pressure resistance of the arms is negligible and does not affect the recording pen. An errant pen is free to exceed both microswitch arms, in either direction, without damage to any components.

The microswitch carriage is geared to a helically cut shaft, and it slides on a guide bar. These components are attached to supporting brackets beyond each end of the chart paper, allowing full-scale travel of the center point of the microswitch carriage. The geared shaft is driven by a motor and gear assembly (called the rate control assembly) mounted at the left side of the recorder (Figure 7 and Appendix II). When the shaft rotates, the microswitch carriage slides across the guide bar. By placing the appropriate gear on the extension of the drive shaft, the speed of the carriage across the temperature scale can be varied at 1, 2, 3, and 4°C per hour. The pen carriage "follows" the microswitch carriage, staying within the bounds of the two microswitches, except for a minimal overtravel. The oscillating deviation of the temperature

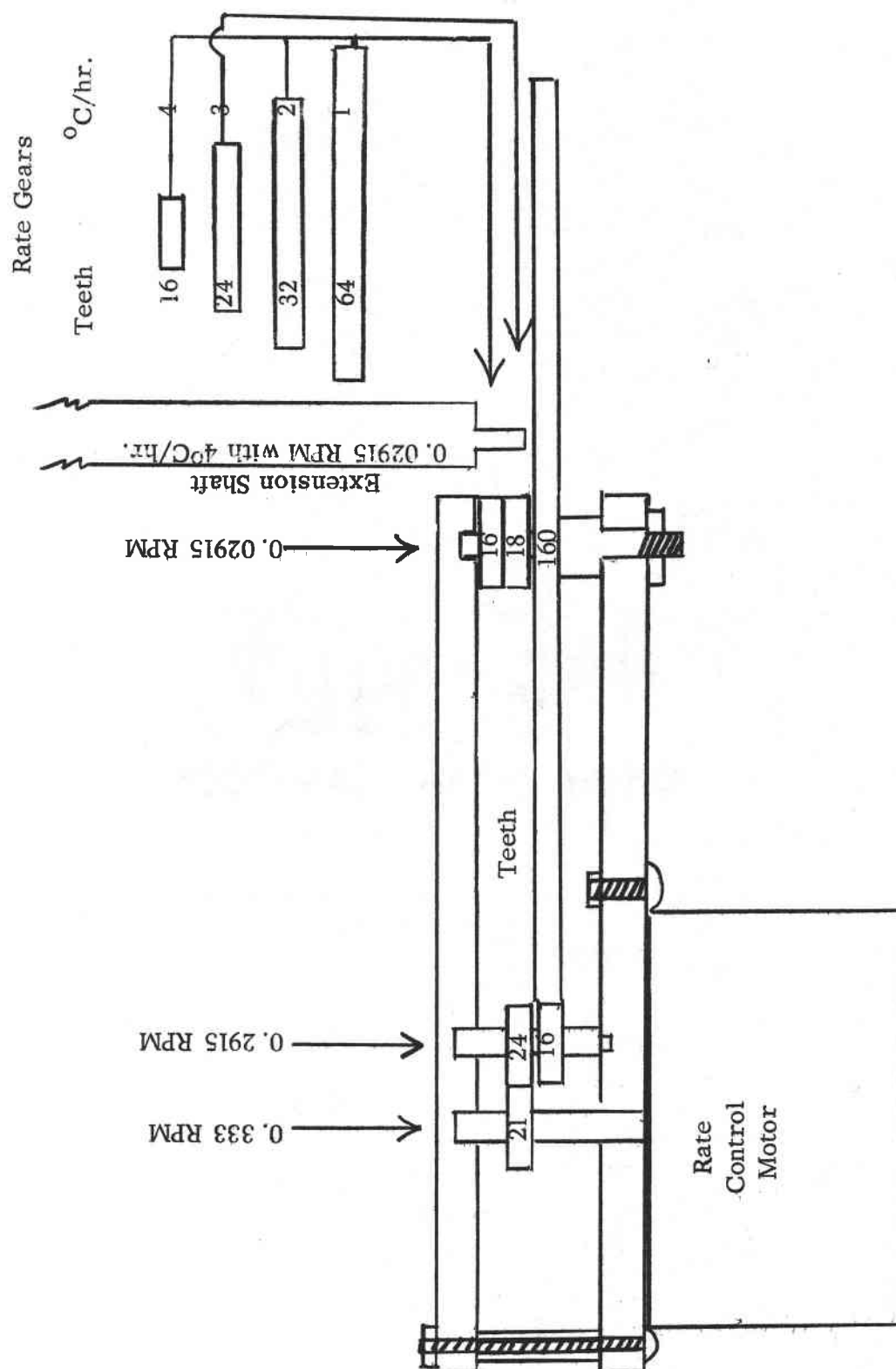


Figure 7. Rate Control Assembly Diagram

from the linear rate will be discussed later.

The versatility of temperature control is achieved by the sequential operation of the reversible rate control motor and a time clock, the control of which are achieved by limit switches (Figure 8). The first step entails setting of the microswitch carriage at -1.5°C , which is the upper temperature limit of the system, as defined by the upper limit switch attached to the recorder at the right edge of the chart paper. With a freezing rate selected and the proper gear in place, the rate control motor drives the carriage across the chart until it reaches the lower limit switch. This switch slides on the guide bar, and is secured by holding notches at each degree from -5°C to -45°C . The microswitch carriage trips the lower limit switch at the temperature set previously, turning the rate control motor off and switching to the time clock, which has been set to a pre-selected temperature holding period up to 24 hours. When this period has elapsed, a specially installed microswitch in the time clock is tripped, turning off the time clock and switching to the reversed direction of the rate control motor, which drives the microswitch carriage back across the scale until the upper limit switch is tripped and the rate control system is turned off. The rate control system has a power switch and directional indicator lights on the control panel. Two additional circuits are employed in the apparatus (Figure 8). Each has a power switch and indicator light on the control panel. The refrigerator circuit is a simple "off-on" system, controlled by the carriage microswitch. The

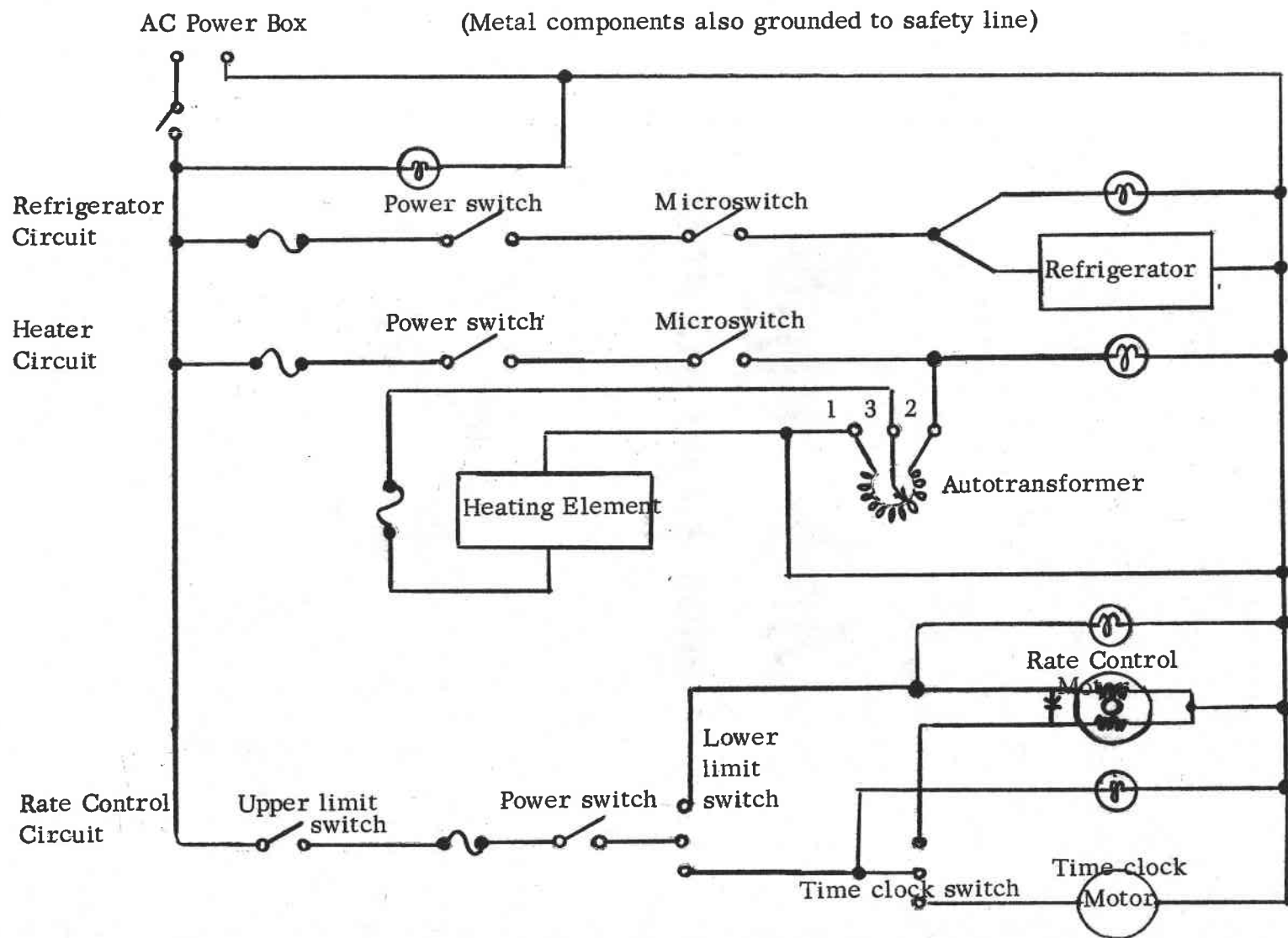


Figure 8. Circuit Diagram

heater/autotransformer circuit is slightly more complex. The direct correlation of the autotransformer to the temperature scale and refrigerator is achieved by a rigid arm connected to the microswitch carriage assembly (Figure 6 and Figure 9). This arm moves across the scale with the carriage. As it moves, the free end slides in a slotted arm which is attached to the autotransformer control shaft. In this way, the linear motion of the pen carriage is translated to the rotary motion of the autotransformer brush, as previously described.

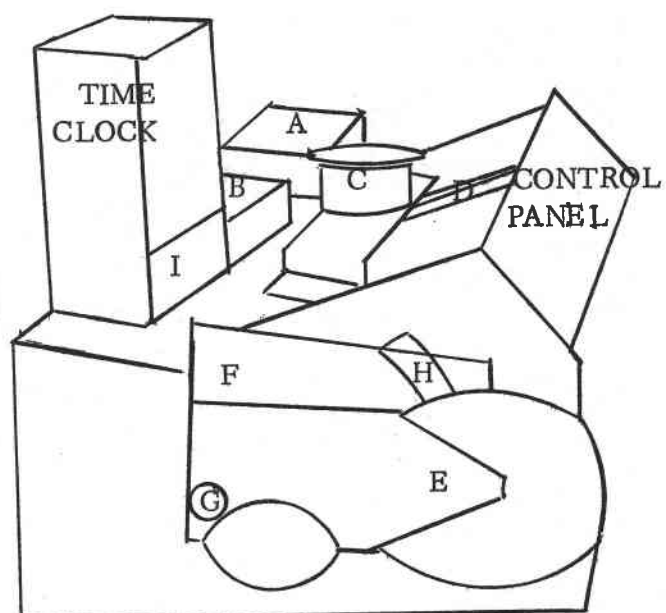
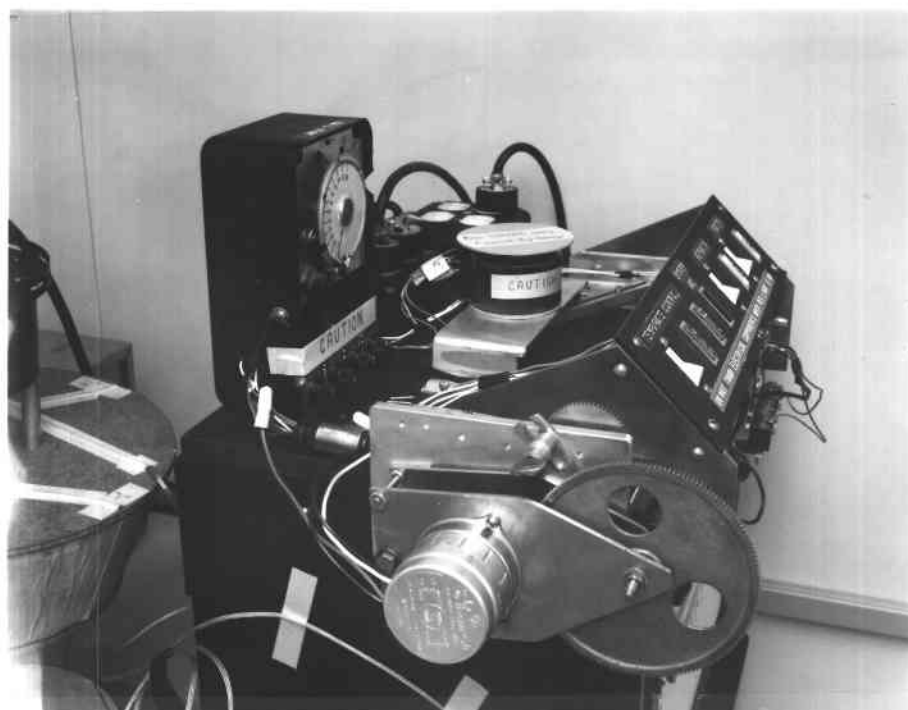


Figure 9A. Top view of the temperature control apparatus

Figure 9B. Diagram of the temperature control apparatus

- A. AC power box with system AC switch
- B. Refrigerator/heater plug-in box
- C. Variable autotransformer
- D. Slotted arm connecting autotransformer to microswitch carriage assembly
- E. Rate control assembly
- F. Mounting block
- G. Assembly pivot
- H. Sliding adjustment for gear accommodation
- I. Fuse panel

RESULTS AND CONCLUSION

Results of Operation

The rate control assembly was tested for accuracy by running the mechanism across the full scale. Since the number of teeth on the 2°C and 4°C gears are whole number multiples of the number on the 1°C gear, accuracy of one means accuracy of the others. Error, proportional to the number of revolutions, is more obvious in a longer trial. In a 45 hour trial of the 1°C gear, 44.8°C change was produced instead of the expected 45°C. The difference is negligible. The 4°C gear produced 42°C change in 10 1/2 hours, for an average of 4°C per hour. The 1, 2, and 4°C gears are considered errorless. The 3°C gear produced an average rate of 2.978°C per hour over a 14 hour period. It is also considered errorless.

The oscillating deviation of the temperature from the linear rate of position change of the microswitch carriage was determined for increasing and decreasing temperatures at 1°C and 4°C per hour. This variation was that allowed as the pen roamed between the bounds established by the microswitches. It was a consistently oscillating motion, alternating high and low around the theoretical linear trace. Maximal variation occurred for the slower rate when temperature was decreased. The faster rate varied most as temperature increased. Variations from held temperatures at various points across the scale were also determined. In all cases, variations were greatest at temperatures below -40°C, where displacement from

ambient temperature is greatest. For this reason, the apparatus is best used at temperatures not less than -40°C . Maximal variation with a decreasing temperature was $\pm 0.4^{\circ}\text{C}$. Maximal variation with an increasing temperature was $\pm 0.6^{\circ}\text{C}$. The holding temperature maximal variation was $\pm 0.3^{\circ}\text{C}$. To these figures must be added the $\pm 0.3^{\circ}\text{C}$ accuracy determined by calibration of the recorder. The overall statement of accuracy is then $\pm 0.9^{\circ}\text{C}$. Freezing and thawing rates are as selected, with achieved temperature at any given point within $\pm 0.9^{\circ}\text{C}$ of the expected temperature.

The reproducibility of these conditions became evident over the period of development of the apparatus. Various changes in switches and auto-transformer settings were the causes of rechecks of variation. At no time were values greater than those stated above for the apparatus in final form.

Variation between test tubes was determined by randomly selecting four tubes for thermocouple insertion. A specially designed, automatic, rotary stepping switch assembly was used to convert the recorder to a twelve-point recorder. Each tube temperature was recorded at three consecutive positions, for a total of approximately 30 seconds. Work with the apparatus indicated that maximum differences could be expected at 4°C per hour. The results showed negligible variation between tubes within and between the two circles of tubes with decreasing and holding temperatures. With increasing temperature, the outer circle proved to be of higher temperature only while temperature changed and only below -40°C . This variation is acceptable.

The final test of the apparatus consisted of a series of runs using one-inch twigs with needles attached. These were cut from a Douglas-fir tree and were homogeneous in age, crown position, and genotype. Sets of four samples each were subjected to similar pre-freezing conditions in a cooler at 2°C. Freezing was at 1°C per hour, holding for two hours, and thawing at 1°C per hour. Over a four-day sampling period in late May, successive runs gave total needle kill at -10°C, no kill at -5°C, no kill at -6°C, and approximately 50 percent kill at -7°C. Determination of kill was made by visual inspection, judging as dead those needles with a dull, gray-green appearance after freezing. A detectable odor was also noted. Control samples were used with all runs. These maintained their glossy green appearance.

Conclusion

From an examination of the freezing process in plant tissue and the nature of freezing injury, a uniform freezing rate, uniform holding temperature, and uniform thawing rate were determined to be important conditions for cold tolerance testing. It is essential that close control be exercised over these conditions if accurate tests are to be performed. Reproducibility is also an essential condition. A control apparatus was designed to satisfy these requirements. It is based on the principle of refrigerator-heater balance to achieve a steady state within the system. This balanced system

is then moved through the desired temperature range at the desired rates and in the desired directions. Testing of the apparatus revealed satisfaction of the basic requirements. The temperature accuracy achieved with the apparatus is sufficient to reveal species differences at the expected cold tolerance values.

BIBLIOGRAPHY

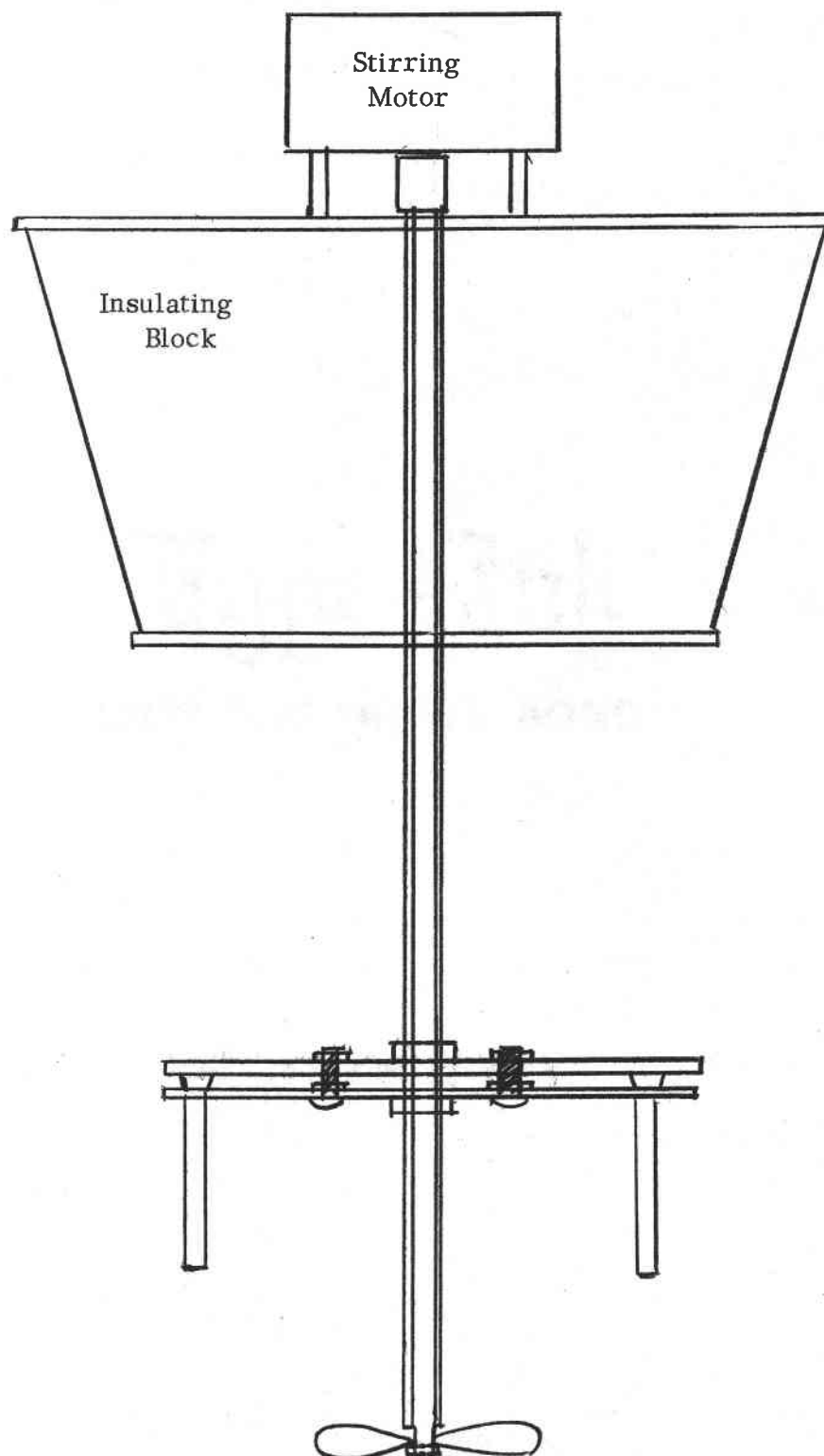
- Chemical Rubber Company. 1947. Handbook of chemistry and physics. 30th ed. Cleveland. 2686 p.
- Dexter, S. T. 1956. The evaluation of crop plants for winter hardiness. *Advances in Agronomy* 8:203-239.
- Eguchi, T. et al. 1966. Studies of selection of frost-hardy cryptomeria. *Silvae Genetica* 15:84-89.
- Glerum, C., J. L. Farrar and R. L. McLure. 1966. A frost hardiness study of six coniferous species. *Forestry Chronicle* 42:69-75.
- Greenham, C. G. and H. Daday. 1960. Further studies on the determination of cold hardiness in Trifolium repens L. and Medicago sativa L. *Australian Journal of Agricultural Research* 11:1-15.
- Havis, J. R. 1964. Freezing of rhododendron leaves. *Proceedings of the American Society of Horticultural Science* 84:570-574.
- Irving, R. M. and F. O. Lanphear. 1967. Environmental control of cold hardiness in woody plants. *Plant Physiology* 42:1191-1196.
- Johansson, N. O. and B. Torssell. 1956. Field trials with a portable refrigerator. *Acta Agriculturae Scandinavica* 6:81-99.
- Levitt, J. 1956. The hardiness of plants. New York, Academic. 278 p.
- _____. 1966. Winter hardiness in plants. In: *Cryobiology*, ed. by H. T. Meryman. London, Academic. p. 495-563.
- Meryman, H. T. 1956. Mechanics of freezing in living cells and tissues. *Science* 124: 515-521.
- _____. 1957. Tissue freezing and local cold injury. *Physiological Reviews* 37:233-251.
- Olien, C. R. 1967. Freezing stresses and survival. *Annual Review of Plant Physiology* 18:387-408.
- Parker, J. 1955. Annual trends in cold hardiness of ponderosa pine and grand fir. *Ecology* 36:377-380.

- _____ 1957. Seasonal changes in some chemical and physical properties of living cells of ponderosa pine and their relation to freezing resistance. *Protoplasma* 48:147-163.
- _____ 1959. Seasonal variations in sugars of conifers with some observations on cold resistance. *Forest Science* 5:56-63.
- _____ 1961. Seasonal changes in cold resistance of some northeastern woody evergreens. *Journal of Forestry* 59:108-111.
- _____ 1963. Cold resistance in woody plants. *Botanical Review* 29:123-201.
- Sakai, A. 1966. Survival of plant tissue at super-low temperatures. IV. Cell survival with rapid cooling and rewarming. *Plant Physiology* 41:1050-1054.
- Salt, R. W. and S. Kaku. 1967. Ice nucleation and propagation in spruce needles. *Canadian Journal of Botany* 45:1335-1346.
- Siminovitch, D. and D. R. Briggs. 1953. Studies in the chemistry of the living bark of the black locust in relation to its frost hardiness. III. The validity of plasmolysis and desiccation tests for determining frost hardiness of bark tissues. *Plant Physiology* 28:15-34.
- Siminovitch, D. et al. 1964. The quantitative estimation of frost injury and resistance in black locust, alfalfa, and wheat tissues by determination of amino acids and other ninhydrin-reacting substances released after thawing. *Canadian Journal of Botany* 42:637-649.
- Takatoi, I., T. Watanabe and U. Kamada. 1966. Frost damage in forest trees. (IX). Frost hardiness in todo fir (*Abies sachalinensis* Fr. Schm.) and white spruce (*Picea glauca* Voss.). In: Annual report of the Forest Experiment Station, Hokkaido, 1965. p. 56-80. (Abstracted in *Forestry Abstracts* 28:3452. 1967).
- White, W. C. and C. J. Weiser. 1964. The relation of tissue desiccation, extreme cold, and rapid temperature fluctuations to winter injury of American Arborvitae. *Proceedings of the American Society of Horticultural Science* 85:554-563.

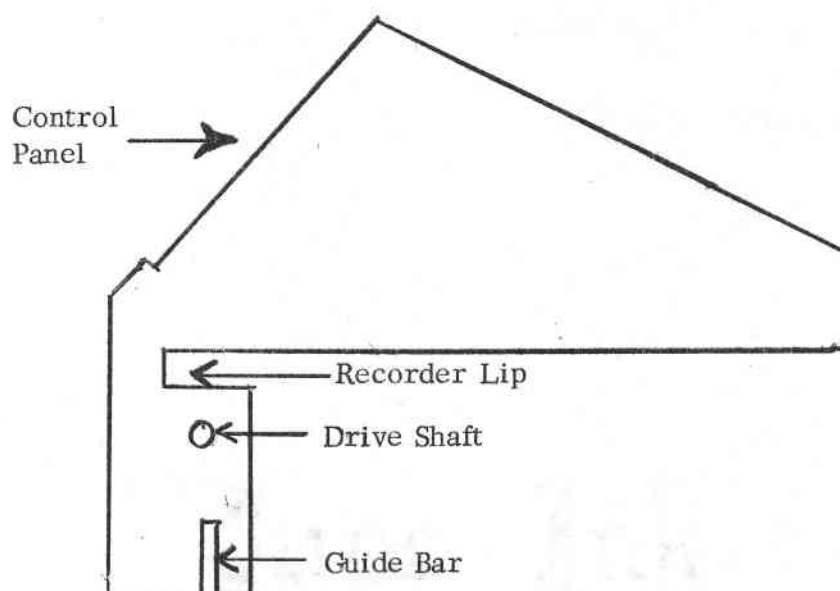
APPENDICES

APPENDIX I. COMPONENTS

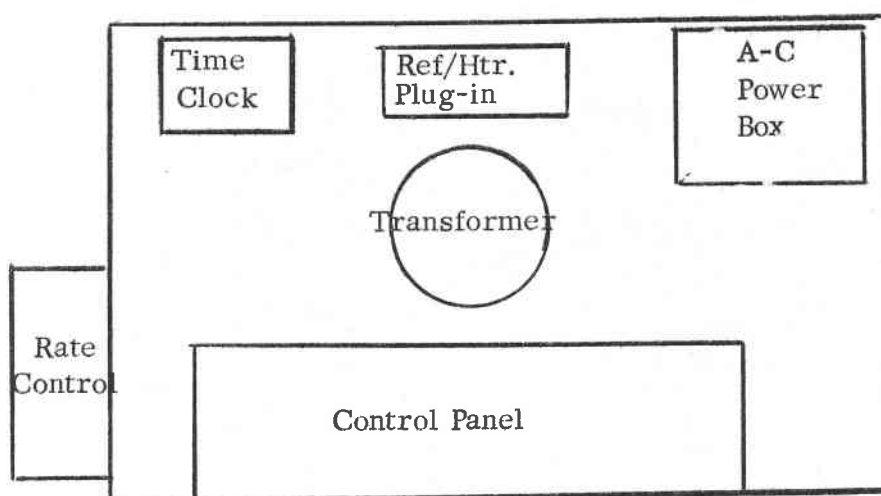
A. Stirring Assembly Diagram



B. Drive Shaft and Guide Bar Support



C. Top view of Control Apparatus



APPENDIX II. LIST OF PARTS

- A. Heating tape, silicone rubber embedded, 115V, 144W max.
(Briscoe Manufacturing Company, Columbus, Ohio, #BS 6 1/2)
- B. Variable autotransformer, 120/132V, 1.75Amp.
(Staco Incorporated, Dayton, Ohio, 175BU)
- C. Synchronous reversible motor, 1/3 RPM, 5W
(Hurst motor, Allied Electronics, #41#7154C)
- D. Miscellaneous lights, switches, etc.
- E. Time clock with microswitch
- F. Gears as noted (specialized to individual need)