Chapter 11

Supercooling and Freezing in Winter Dormant Animals

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William Schmid received his B.S. in Wildlife Management from the University of Minnesota (1959) and his Ph.D. in Zoology from the University of Minnesota (1962). He currently is a Professor in the Department of Ecology and Behavioral Biology at the University of Minnesota. His current research interests include studying the seasonal patterns of supercooling and production of cyroprotectant or antifreeze compounds in the red spider mite (Trombidium), and the role of white-footed mice (Peromyscus leucopus) as host for the tick vector (Ixodes dammini) which harbors the spirochete (Borellia burgdorferi) of Lyme disease. He has a 1987-88 National Research Council Research Fellowship, and will be a USA-USSR Interacademy exhange fellow, September 1 through December 15, 1987. He also received a Fulbright Lectureship, Novosibirsk State University, Academgorodok, USSR, March 1 through June 1, 1988.

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Winter ecology is an extensive field of investigation that includes meteorology, microclimatology, phenology, natural history, physiology and biochemistry. Many organisms have special morphological, physiological and behavioral adaptations which are necessary for survival in seasonally cold regions. Organisms that live in such regions have four general categories of adaptation to winter stresses: (1) die, (2) migrate, (3) remain active and (4) become dormant. Death, migration and winter activity are all adequate adaptations to the winter cold season, but it is winter inactivity or dormancy of many animals that has been the focus of research in my laboratory during the past decade. Animals in a state of winter torpor are in one of two groups: (1) they can survive freezing (frost tolerant), or (2) will die if ice forms within their bodies so that they develop antifreeze chemicals to depress their freezing points and deeply supercool (frost resistant).

A small land snail, *Vallonia perspectiva,* is an example of the latter case. The cooling and freezing curve for a single specimen is shown in Fig. 1 which illustrates the application of thermocouple thermometry. The phenology of supercooling by this species is shown in Fig. 2 where the winter specimens are seen to supercool to much lower temperatures than those of summer. On the other hand, if we measure supercooling points of frost tolerant animals it is usually found that internal ice formation occurs at higher temperatures during the winter. A summary of supercooling point measurements is given for a variety of Minnesota species in Table 1. In all but one case, *Mordellistina unicolor,* frost tolerance is associated with relatively warm supercooling temperatures; *i.e.,* those species which can survive freezing usually promote internal ice formation during the winter season. The examples provided here illustrate applications of the thermometry techniques to be described now.

The goldenrod gallfly, *Eurosta solidagensis,* will be used to demonstrate the techniques of thermometry as applied to studies of supercooling and freezing in winter dormant organisms. The gallfly is distributed widely across the eastern United States and winters as a third instar larva in diapause within the ball galls of goldenrod *(Solidago sp.)* plants. Third instar larvae collected in early fall will typically supercool to -15 °C., and do not survive freezing. However when these larvae are collected in midwinter they will supercool to -9 °C., and survive freezing (frost tolerant). Ball galls can be collected in midwinter and stored in a freezer until needed for laboratory measurement of supercooling points of the resident larvae. Ladybird beetles, *Hippodamia convergens,* in winter diapause, obtained from commercial suppliers, can be used to the demonstrate deep supercooling (frost resistance) adaptation to winter cold exposure. It is important to keep in mind that new, original discoveries can be made with these methods by utilization of local organisms which have never been studied for their adaptations to winter survival.

The term "freezing" simply indicates the formation of ice: phase change from liquid water to solid ice as temperature drops. "Supercooling" on the other hand refers to a

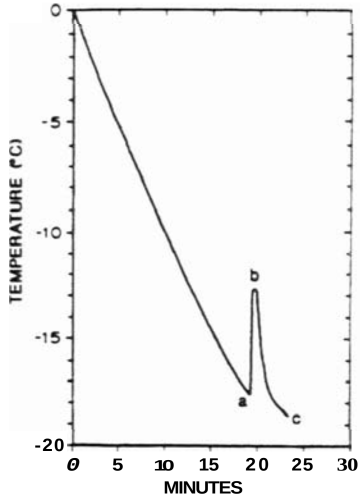


Fig. 1. Cooling and freezing curve for a terrestrial snail, Vallonia perspecriva, collected from its wintering site in January. 1981. Point (a) is the supercooling point (–17.6 º C), the sudden rise in specimen temperature from a to b is due to exothermic release of heat by internal ice formation. and freezing continued from b to c. The snail did not survive freezing. After Schmid (1986).

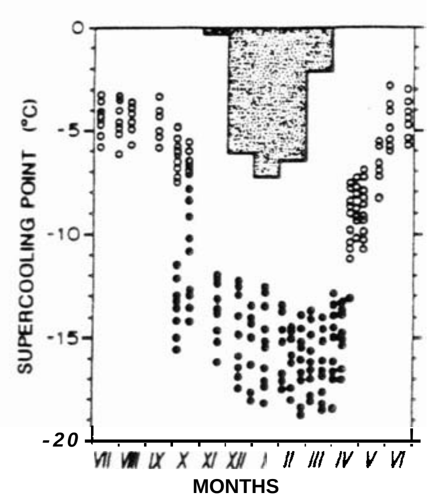


Fig. 2. The annual cycle of supercooling points for Vallonia perspecriva. The open circles (o) represent active specimens and the closed circles (e) represent inactive animals with epiphragms over the aperature of their shells. At no time during the year could this snail survive if frozen. Stippled bars at the top of the graph represent the coldest microclimate temperature of each month through the winter season After Schmid (1986).

Table 1. Supercooling and Frost Tolerance of Some Minnesota Animals in the Winter

SPECIES		SCP.	(ºC)	N	Survival	Winter Habitat
Amphibians						
Rana septentrionalis	**Mink Frog**	-2.7	±.3	4	0	**Aquatic**
Rana pipiens	Leopard Frog	-2.6	±.4	3	0	Aquatic
Rana sylvatica	**Wood** Frog	-1.9	±.3	6	6	Terrestrial
Hyla versicolor	Grey **Tree** Frog	-2.1	±.3	8	8	Terrestrial
Hyla chrysoscelis	Green **Tree** Frog	-2.0	±.3	4	4	Terrestrial
Hyla crucifer	**Spring** Peeper	-2.0	±.2	4	4	Terrestrial
Bufo americanus	**American** Toad	-1.8	±.4	4	0	Terrestrial
Ambystoma laterale	Jefferson **Salamander**	-3.4	±.1	6	0	Terrestrial
Snails						
Vallonia perspectiva	Land Snail	-15.4	±1.8	84	0	Terrestrial
Gastrocopta armifera	Land **Snail**	-30.6	±2.3	52	0	**Terrestrial**
Insects						
Mordellistena unicolor	Gall **Beetle**	-35.2	±3.9	10	2	Terrestrial
Eurosta solidagensis	Goldenrod **Gallfly**	-8.9	±1.5	27	27	Terrestrial
Achorutes nivicolus	Snow Flea	-19.2	±1.1	18	0	Terrestrial
Platypsylla castoris	Beaver Beetle	-17.6	±1.8	5	0	Terrestrial
Ticks						
Dermacentor variabilis	**Wood** Tick	–14.2	±1.6	15	0	**Terrestrial**
Dermacentor albipictus	Winter Tick	–17.4	±2.5	9	0	**Terrestrial**
Ixodes dammini	Deer **Tick**	–16.3	±2.4	10	0	Terrestrial
Mites						
Trombidium sp.	Red Velvet **Mite**	–7.4	±.6	11	11	Terrestrial
Spiders						
Dictyna sp.	Spider	–32.1	±.7	8	0	Terrestrial
Metaphidippus sp.	**Jumping** Spiders	–23.8	±2.3	38	0	**Terrestrial**

After Schmid (1986)

freezing temperature below what is expected from the solute (colligative effect) concentrations in the aqueous solution of organisms. It is the temperature of spontaneous ice formation seen in intact animals as the temperature is gradually lowered. For this reason surface contact thermometry is necessary. Invasive placement of temperature sensors might provide artificial sites for ice nucleation that are not present in the intact animal. Although both thermistors and thermocouples are adequate choices of temperature sensors, the latter will be described and used in this exercise. In addition to the specimen cooling system, measurement of supercooling and freezing requires a sensor, a detector (high input impedance microvoltmeter for amplification) and a recorder.

The **COOLING SYSTEM** consists of a liquid bath into which a test tube containing the specimen and sensor can be placed for removal of heat. In the simplest case, this might be a thermos filled with a solution of automotive antifreeze which has been left in a deep freeze overnight. A dewar flask filled with antifreeze solution or ethyl alcohol can be cooled by addition of chips of dry ice. For shallow cooling temperatures, a styrofoam chest filled with crushed ice and rock salt might be sufficient. Controlled cooling baths, programmed for specific cooling rates and temperatures are commercially available and usually more convenient than these simple systems; e.g., Neslab or Forma. The choice of cooling system will depend upon your specific needs and budget available for equipment expenditures.

The **SENSOR** for continuous measurement of specimen temperature is constructed of fine (.002 inch diameter = 3 mil) thermocouple wires. Type T or copper-constantan wires are readily available and easily fabricated into sensors for contact thermometry. If the reference junction of the thermocouple pair is held at zero degrees C. in a bath of chipped ice and distilled water, the thermoelectric voltage can be easily converted to temperature by values given in Table 2. For example, if a cooled specimen had an exotherm at -.668 mV, its supercooling point would convert to -17.6 degrees C.

Table 2. Thermoelectric voltage for copper-constantan (Type T) thermocouples.

DEG C	0	1	2	3	4	5	6	7	8	9	10	OEG C
-90	-3.089	-3.118	-3.147	-3.177	-3.206	-3.235	-3.264	-3.293	-3.321	-3.350	-3.378	-90
-80	-2.788	-2.818	-2.849	-2.879	-2.909	-2.939	-2.970	-2.999	-3.029	-3.059	-3.089	-80
-70	-2.475	-2.507	-2.539	-2.570	-2.602	-2.633	-2.664	-2.695	-2.726	-2.757	-2.788	-70
-60	-2.152	-2.185	-2.218	-2.250	-2.283	-2.315	-2.348	-2.380	-2.412	-2.444	-2.475	-60
-50	-1.819	-1.853	-1.886	-1.920	-1.953	-1.987	-2.020	-2.053	-2.087	-2.120	-2.152	-50
-40	-1.475	-1.510	-1.544	-1.579	-1.614	-1.648	-1.682	-1.717	-1.751	-1.785	-1.819	-40
-30	-1.121	-1.157	-1.192	-1.228	-1.263	-1.299	-1.334	-1.370	-1.405	-1.440	-1.475	-30
-20	-0.757	-0.794	-0.830	-0.867	-0.903	-0.940	-0.976	-1.013	-1.049	-1.085	-1.121	-20
-10	-0.383	-0.421	-0.458	-0.496	-0.534	-0.571	-0.608	-0.646	-0.683	-0.720	-0.757	-10
0	0.000	-0.039	-0.077	-0.116	-0.154	-0.193	-0.231	-0.269	-0.307	-0.345	-0.383	0
0	0.000	0.039	0.078	0.117	0.156	0.195	0.234	0.273	0.312	0.351	0.391	0
10	0.391	0.630	0.470	0.510	0.549	0.589	0.629	0.669	0.709	0.749	0.789	10
20	0.789	0.830	0.870	0.911	0.951	0.992	1.032	1.073	1.114	1.155	1.196	20
30	1.196	1.237	1.279	1.320	1.361	1.403	1.444	1.486	1.528	1.569	1.611	30
40	1.611	1.653	1.695	1.738	1 7 8 0	1.822	1.865	1.907	1.950	1.992	2.035	40

The fine wires of the sensor or measurement junction of the thermocouple are soldered to heavier (24 AWG) copper-constantan lead wires for hook-up to the microvoltmeter or amplifier. The heavier lead wires are also used to form the reference junction where temperature is constant and problems of thermal inertia are not important. The fine wires of the sensor junction are necessary because they have very low heat capacity or thermal inertia necessary to detect the heat released (latent heat of fusion of water) when ice forms inside of a specimen at its supercooling point. Structural durability for the fine. 2 mil sensor wires can be improved by using wire with a teflon sheath for insulation: the teflon covering provides strength as well as insulation. The insulation can be burned from the end of the wires which can then be scraped clean and twisted together to form electrical contact for the sensor junction. For greater integrity of the sensor thermocouple junction, the twisted wires can be wiped with solder. This junction must be held in close contact with the specimen as it is cooled.

A 15 ml conical centrifuge tube with the fine wire sensor junction inside at the bottom of the cone is a very simple system into which a specimen can be placed for cooling and measurement of its supercooling point. A more reliable system, required for small specimens can be constructed from a piece of plastic rod. The rod is cut into halves along its longitudinal axis (through its diameter) and the thermocouple sensor wires are glued onto the flat cut surface. A specimen is positioned against the sensor junction and held in place with a foam rubber pad that is itself taped to the plastic rod. The rod is then placed in a test tube and lowered into the cooling bath. Variations on the construction of the sensor junction and specimen holder must be made to accommodate the organism under study and the cooling rates desired. For example, added insulation might be necessary to control the cooling rate in the absence of a programmed cooling bath.

The **DETECTOR-RECORDER** operate as a unit. The sensitivity and amplification of the detector and the sensitivity of the recorder are adjusted to give readout precision to accurately detect the thermoelectric voltage change at supercooling. Two detectors of thermoelectric voltage that I have used successfully are the Keithly 155 microvoltmeter and the Omega 2809 digital thermometer. Both instruments have the necessary high input impedance and output connections to a recorder. Both the Gould 105 and the Fisher Recordall 5000 have been satisfactory recording devices for continuous records of temperature change. It is certainly feasible to use other combinations of devices for detection and recording of thermoelectric voltages from a Type T thermocouple sensing system; e.g., an A-D card in a personal computer might be used to capture the record of thermoelectric voltage change in digital form for later printout to hard copy. The sensitivities of the detector and recorder can be adjusted to provide the most useful scale on final output of strip chart paper. If the minimum temperature records do not go below -25 degrees C., adjustment of sensitivities to give full scale recorder pen deflection of 0 to -1 mV will be easily translated to a scale of temperature. Likewise, a full scale pen deflection of 0 to -2 mV will accommodate cooling to about -55 degrees C. Such systems of sensor-detector-recorder with subzero cooling can be used to examine many different examples of organism-cold season adaptation seen in patterns of supercooling and freezing. The experimental cooling rates used are best defined by knowledge of normal variations in temperature which are experienced by organisms in nature (Figs. 3-5). A nominal cooling rate often used in experiments of supercooling of insects is -1 degree C. per minute. The effects of cooling rate upon measured supercooling point and upon freezing survival should always be checked as a source of experimental bias. Biochemical changes accompany the seasonal patterns of supercooling and some of these can be easily detected by relatively simple methods.

Paper chromatography will detect polyhydric alcohols which are often associated with seasonal development of freeze tolerance or deep supercooling. A single gallfly larva, after measurement of its supercooling point, is homogenized in .5 ml of cold 70% ethanol. This crude extract is centrifuged to settle tissue debris and the

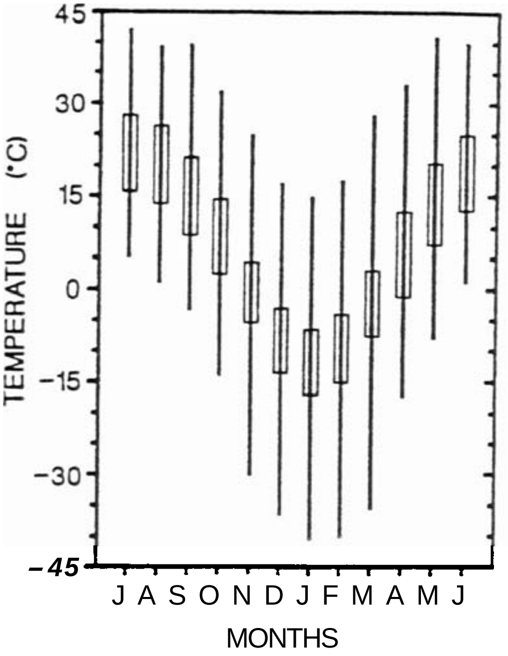


Fig. 3 Long term weather station records for the Twin Cities. The open bars represent average monthly low and high temperatures, while the lines are record highs and lows.

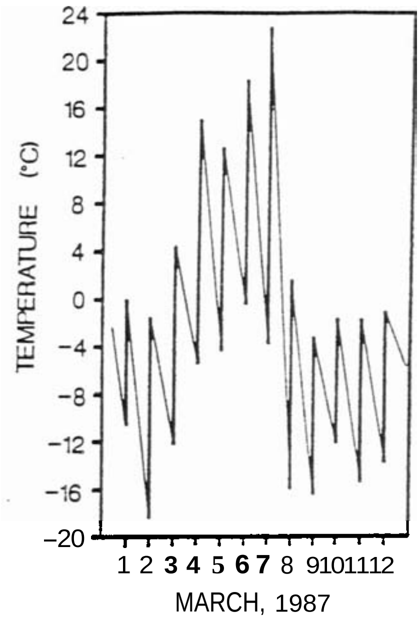


Fig. 4 Daily high and low temperatures recorded at the University of Minnesota Forestry and Biology Station.

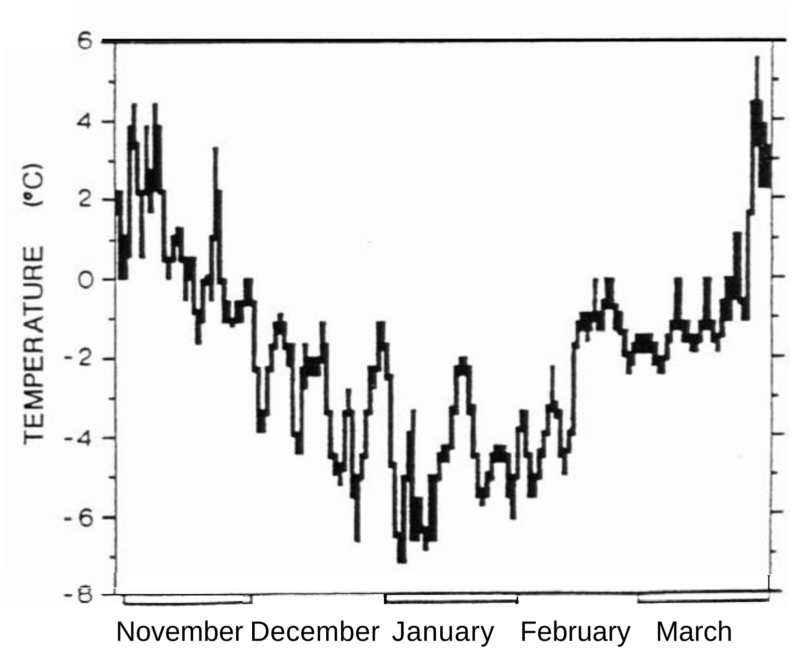


Fig. *5* Microclimate temperature variation recorded through the winter (1980-81) at the soil surface, beneath leaf litter, in Elm Creek Park Reserve, Hennepin County, Minnesota.

supernatant is saved. Aliquots of 5 uL are spotted onto Whatman No. 1 paper along with mixed standards (5 uL of .1 M) glycerol, sorbitol and trehalose. Ascending chromatograms are developed in a solvent system of n-butanol:pyridine:water = 6:4:3 for about seven hours. The chromatograms are dried and sprayed with periodate and iodine-starch as described by Somme (1964). The developed chromatogram is dried and sprayed with 0.01 M aqueous solution (.23 g/100 ml) of potassium periodate and dried again. Then it is sprayed with the iodine-starch solution (35% saturated sodium tetraborate, .3% potassium iodide, .9% boric acid and 2% soluble starch) and dried. Glycerol and sorbitol will appear as white spots on a dark blue background, while trehalose appears as a light gray smudge on the same background. Chromatographic separation (migration) is approximately proportional to molecular weight so that glycerol is closest to the solvent front and trehalose is farthest away. The amount of each compound can be estimated by the size (area) of each spot. It is necessary to run standards of different concentrations in order to estimate concentration by spot area. Thin layer chromatography and high performance liquid chromatography are obvious alternatives to this relatively crude method of analysis. Nuclear magnetic resonance is also being used to estimate the seasonal production of cryoprotectant and/or antifreeze biochemicals by dormant animals of seasonally cold regions.

The materials and methods described in this brief presentation can be used to illustrate adaptations for survival during cold seasons of the year in laboratory courses of animal and plant physiology as well as physiological ecology. However, students with interest, curiosity and scientific aptitude will be able to use this exercise as a guide for original research wherein their findings will provide us with new and useful information about the adaptations of organisms to their environment. I close this discussion by thanking three people who have helped me develop my interest and skill to examine problems of cold temperature adaptation: Wally Saatala, Bob Maxwell and Rick Lee.

Appendix 1: Supplies

Suppliers of general laboratory equipment:
Fisher Scientific
50 Fadem Road
Springfield, NJ 07081

Cole-Parmer Instrument Company
7425 North Oak Park Ave.
Chicago, IL 60648

Bio Rad Laboratories
32nd and Griffin
Richmond. CA

Supplier of low temperature controlled baths:
Neslab Instruments, Inc.
871 Islington St.
Portsmouth, NH 03801

Supplier of biochemical standards for paper chromatography:
Sigma Chemical Co.
P. O. Box 14508
St. Louis, MO 63178

Suppliers of thermometry equipment and supplies:
Omega Engineering, Inc.
One Omega Drive
Box 4047
Stamford, CT 06907

Keithly Instruments
Cleveland, OH 44139

California Fine Wire Co.
Grover City, CA

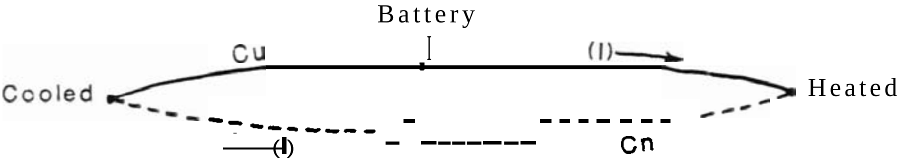
Thermometries
808 U.S. Highway I
Edison, NJ 08817

Supplier of specimens for supercooling study:
Fountain's Sierra Bug Co.
P.O. Box 114
Rough and Ready, CA 95975
Source of ladybird beetles, *Hippodamia convergens*, in diapause.
About $10 per quart

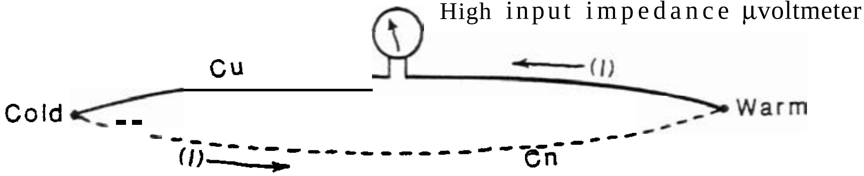
There are certainly alternative sources to those above, but this listing gives you someplace to start. There is no intention of special endorsement for any of these suppliers.

Appendix 2: Thermocouples

Thermocouples generate a thermoelectric voltage whenever there is a temperature difference between the junctions of the two dissimilar metals of their construction. Thermoelectric describes the phenomenon whereby heat energy is transformed (transduced) to measureable electrical energy, and vice versa. Although the irreversible conversion of electrical current into heat (i.e., heat = I^2R) comes under the category of thermoelectric, it is the reversible phenomenon that we are more concerned with in regard to measurement of environmental or biological temperatures. The Peltier effect: Junctions of dissimilar metals are heated or cooled depending upon the direction in which a current passes through them. This effect is proportional to the first power of current and not to I^2 as in the case of irreversible heating of conductors.



The Thompson effect: An emf (voltage) will arise within a single conductor wherever a temperature gradient is present. The total Thompson effect along a conductor depends only upon the temperature difference between the two ends of the conductor. The Seebeck effect is the algebraic sum of the Peltier and Thompson effects, and it is this total effect that is useful to us in application of thermocouples to thermometry. An electric current and its emf are present in a series of two different metals if the two junctions are at different temperatures; e.g., copper and constantan, where constantan (Cn) is an alloy of 60% copper and 40% nickel.



Thermoelectric sensitivity, dV/dT (µV/ºC) of thermocouples made of the following materials with platinum (reference junction = O^{o}C):

Antimony	47	Aluminum	3.5
Nichrome	25	Platinum	0.0
Iron	18.5	Nickel	-15
Copper	6.5	Bismuth	-72
Silver	6.5	Constantan	-35

So the expected thermoelectric emf from copper-constantan (Cu-Cn) or the Type T thermocouple is: (+6.5) - (-35) = 41.5 µV/ºC). Table 2 provides a listing of thermoelectric voltages for the Type T thermocouples, and it can be seen that the emf per degree Celsius change is very close to the theoretical and quite linear over a wide temperature range.

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