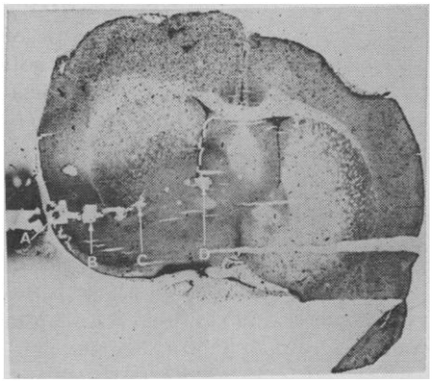

Fig. 2. Coronal section of rat brain after application of probe. Black crater outside brain represents background probe. Some fine dust from the sparking electrodes has fallen on the section. *A*, Outer cortex; *B*, inner cortex; *C*, nucleus caudate-putamen; *D*, tip of ventricle.

with caution. The pure graphite used in spectroscopic electrodes is the best support for samples, as can be seen from examining the spectrum of the "chemically clean" ordinary glass slides in Fig. 1. Direct electronic readout permits rapidity of analysis for the selected elements when many samples of the same matrix are to be analyzed. Photographic spectra used for the illustrations presented here, however, afford a greater versatility in the number of atomic elements that may be identified in each sample.

Until the advent of the laser, no practical method for *in situ* spectroscopic analysis existed because selection from intact sections was not possible without considerable tedious manipulation. The technique described is only an illustration of the possibilities, which include intracellular probes, analysis from frozen sections, and extension of the spectrum to higher and lower frequency ranges (*3*).

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7 August 1963

Freezing-Point Depression: New Method for Measuring Ultramicro Quantities of Fluids

Abstract. *Accurate determination of the solute content of millimicroliter samples of biological fluids can conveniently be made with an instrument consisting of a thermoelectric cooler controlled by an electronic proportional servo system. Accuracies to within less than 2 percent have been achieved in determining the osmolarity of body fluids.*

Freezing-point depression of millimicroliter samples of biological fluids can be determined conveniently and accurately by a new method. Although methods now exist for making these determinations, their dependence upon solid refrigerants, large regulated baths, and expanded-scale thermometers makes them cumbersome. By using modern materials and electronic techniques, we have eliminated these inconveniences. Thermoelectric junctions provide direct cooling of the samples, so that freezing is accomplished without external coolants. A high-gain electronic servo system senses the temperature of the samples and provides proportional control of the current to the thermoelectric junctions to obtain precise control of the temperature of the samples. The method is convenient because (i) several determinations are made rapidly during a single run; (ii) samples are frozen and, if necessary, refrozen by flipping a switch; and (iii) temperature is controlled to 0.001°C by turning a dial. Accuracy is achieved by making simultaneous determinations on unknowns and standards, finding values for unknowns by interpolating between accurate values for standards, and eliminating problems of thermal equilibrium through tight feedback control of the temperature of the samples. In addition to finding wide use as a freezing point apparatus, the device can be used to control precisely the temperature of ultramicro quantities of sample and thus is suitable for use in controlled-temperature studies of the physiology of microorganisms and cells.

Measurement of freezing-point depression provides a practical means of determining the effective osmotic activity of biological fluids obtained in studies of diffusion, secretion, and absorption in biological organisms. Often, in such studies, minute regions of the organisms are being analyzed; hence it may be impossible or unwise to take samples of fluid larger than 1 millimicroliter. In physiological studies of renal tubular function, for example, samples of this order are obtained, by means of micropuncture techniques, for several kinds of analyses as well as for the freezing-point determinations. The accuracy of the other chemical and physical determinations made during such studies demands that measurement of osmotic pressure be accurate to within a few milliosmoles (*1*).

With current techniques (*2*) the freezing points of individual, micro samples are determined by observing, on an expanded-scale thermometer, the temperature of a regulated bath as the melting of the last ice crystal in the sample is observed through a microscope. With such methods it is necessary to approach the end point slowly to prevent the building up of thermal gradients between the thermometer, the bath, and the sample. Gradients which contribute less than the acceptable error can be tolerated, but strict adherence to laboratory procedure is required to obtain high accuracy.

The method outlined here was designed for handling multiple samples and standards identically and simultaneously so as to decrease the time required per determination, to achieve greater accuracy through close interpolation of unknowns between standards, and to eliminate the necessity of bringing a large bath to thermal equilibrium. In this method, the values obtained for unknown solutions are interpolated between the values for standard solutions maintained in the same environment, so that the accuracy of the values for the standard solutions determines the accuracy of the calibration curve. Equilibrium between the temperature of the sample and the temperature of the control system is obtained by tight thermal control of the sample stage of the instrument. In this way the end point can be approached rapidly, and the temperature can be controlled to make the final ice crystal of a sample shrink, grow, or remain constant in size in instantaneous response to a change of setting of a few divisions on a 1000-division potentiometer.

The instrument is an electronically controlled thermoelectric cooler capable of controlling the temperature of several samples of millimicroliter quantity to within 0.001°C and small enough to be placed in the substage region of a microscope. The cooler utilizes semiconductor thermoelectric

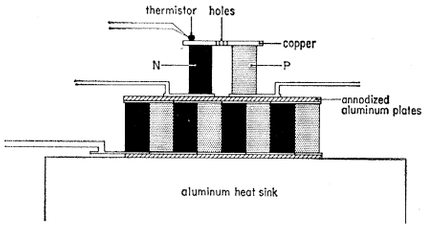

Fig. 1. Thermoelectric cooler assembly, showing details of construction of the top and bottom stages.

couples which, due to the inverse thermocouple effect, or Peltier effect, establish a temperature difference proportional to the current that passes through them (*3*). The cooler, a two-stage device, consists of a high-current bottom stage, for gross cooling, which is thermally in series with a low-current, low-mass top stage for the precise control of the temperature (Fig. 1). The bottom stage, a commercial eight-couple, 15-ampere module (*4*), receives a low-ripple constant current from a power supply with full wave-bridge rectification and choke filtering. In contact with the cold junction of this stage is the hot junction of a single thermoelectric couple requiring a maximum current of 3 amperes at 0.15 volt. Because it is a single couple with low thermal and electrical mass, its cold-junction temperature responds very rapidly to a small change in current and can be set and maintained at a desired temperature through precise control of the current through the couple. The copper bridge which forms the cold junction of the top stage contains eight holes of 0.1-mm diameter,

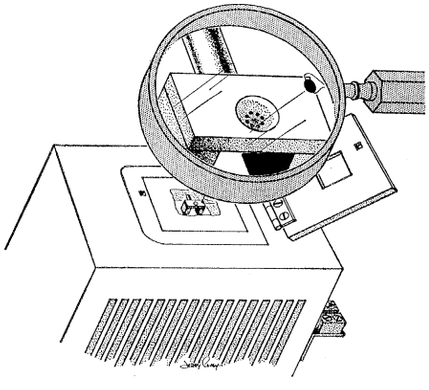

Fig. 2. View of the cooling unit, showing the finned heat sink, the hinged lid, and placement of the thermoelectric cooler assembly. The magnified area shows details of the sample holes, of the depression for oil, and of the thermistor on the sample stage.

arranged around the circumference of a circle 1.4 mm in diameter (Figs. 1 and 2). These holes are for holding the samples (of unknown solutions and of standards) whose freezing points are to be measured. In intimate contact with this copper bridge is a small bead thermistor, which senses the temperature of the copper bridge and forms the input to the proportional controller.

The entire cooling assembly shown in Fig. 1 is recessed into a finned aluminum heat sink (Fig. 2), which is cooled by a small blower. The hot junctions of the bottom stage are in thermal contact with the aluminum, but the rest of the assembly is insulated from it by epoxy foam. A hinged lid containing a glass window can be closed to isolate the freezing stage still further. Additional protection from condensation and frosting on the freezing surface is afforded by purging the enclosed area with dry gas. A light pipe and electrical leads for the two cooling stages and the thermistor enter through sealed holes in the heat sink.

Because of its size (10.1 by 7.6 by 7.6 cm), the entire cooling unit (Fig. 2) can be placed in the substage region of a microscope, so that the determinations can be observed visually. The samples are illuminated from underneath by means of a light-pipe, which transmits light from an outside source. A stereomicroscope with a magnifying power of 45 is suitable for loading the samples, while a power of 112.5 is ideal for making precise determinations on samples of millimicroliter size.

Figure 3 shows the configuration of the basic units of the electronic servo system. The thermistor, which monitors the temperature of the samples, is a negative-temperature-coefficient resistance device which provides an increase in resistance of 3.9 percent in response to a decrease in temperature of 1°C. This device is one leg of an alternating-current bridge, the opposing leg of which consists of variable resistances for temperature selection. The output of this bridge is an error signal proportional to the difference between the temperature of the copper bridge sensed by the thermistor and the temperature selected on the variable resistance dials. The next unit after the bridge is a high-gain alternating-current amplifier with a power-output stage. The amplified signal from this circuit controls the output of a solid-state synchronous rectifier, which supplies to the cooler a low-ripple direct current of the magnitude necessary to bring the temperature of the samples to the desired value and to keep it there.

The proportional controller provides the top stage of the cooler with an amount of current proportional to the deviation of the temperature of the samples from the selected temperature. By adjusting the gain of the system it is possible to choose the amount of deviation which will cause the controller to supply maximum current to the cooler. For any deviation less than this amount, the controller will supply proportionally less current. Thus, when a lower temperature is selected on the variable resistance dials, the controller instantaneously supplies the cooler with a large pulse of current. However, as the temperature of the samples approaches the temperature set on the dials, the current falls off rapidly, becomes zero when the temperature reaches the selected value, and then increases to an amount necessary to maintain that temperature. In this way, the system senses an extremely small deviation from the dial settings, and thus the operator can precisely control the temperature of the samples by turning a dial.

The handling of samples of unknowns and standards requires only standard micropipetting techniques. Micropipets with 5- to 10-micron lumens are used to transfer the samples into the holes. Since the freezing point is not affected by small differences in volume, precise measurement of volume is not necessary. Because samples of this size must be protected from contamination and evaporation, the samples are kept under oil at all times. For this reason, the holes in the copper bridge of the top stage are filled with castor oil, a viscous oil of low freezing point, before the samples are introduced. The samples of unknowns and standards are then pipetted into the holes, where they appear as transparent spheres. To insure that all of the unknowns and standards are in the same environment, the depression in the copper bridge of the top stage (Fig. 2) is filled with oil, so that the samples are all immersed in a common medium.

After the samples have been introduced, the area is purged with dry gas, and the hinged lid is secured. Application of current to the bottom stage for about 2 minutes now brings the temperature of the sample stage to below 0°C. Maximum current is then applied

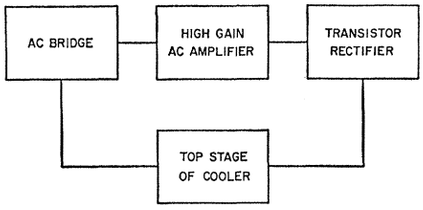

Fig. 3. Block diagram of the proportional controller.

to the top stage, freezing the samples in approximately 30 seconds. Because the temperature is dropped to near −50°C in such a short time, supercooling effects are overcome and the samples are frozen without seeding. In the frozen state, the samples appear as irregular, cloudy spheres. Once the samples are frozen, the proportional controller is switched in, and the temperature of the sample stage is raised to the value set on the dial. As the samples thaw, the solid ice breaks up into smaller crystals, and the operator, who sees all eight samples (standards and unknowns) at once, can easily judge which sample will thaw first. He then uses the dials to control the rate of thawing of the crystals in this sample until one crystal remains. Each dial consists of a stepping switch for coarse control, in series with a ten-turn potentiometer which divides each step of the coarse control into 1000 divisions. The coarse control is used to raise the temperature until one of the samples starts to thaw, and the fine dial gives the operator precise control allowing him to control completely the thawing of the last crystal. Here, the operator is an integral part of the control system. By observing the thawing process and setting the dials accordingly, he carefully regulates the freezing and thawing of the crystals and determines the point at which the last one disappears. This point is defined as the freezing point of the solution. The fact that the final ice crystal can be made to stop thawing and to grow or remain of constant size indicates that it is in temperature equilibrium with the system. If it were not, gradients would exist and heat flow would cause the crystal to change size.

Table 1. Data from determinations on samples of human blood plasma.

Sample No.	Micro method		Macro method
	Millios-moles/kg of H_2O	Error* (%)	Millios-moles/kg of H_2O
1	291	1.04	288
2	279	0.71	281
3	300	.67	298
4	293	4.65	280
5	285	1.73	290
6	277	3.50	287
7	277	1.42	281
8	302	2.72	294
9	286	0.0	286

* Average error, 1.83.

Because of the precision required, accurate standards are used to establish calibration points; values for unknowns between these points are found by interpolation. Since curves for the data obtained are linear over a wide range of osmolalities, the inclusion of two or three standards on each run is sufficient. This method of run-to-run calibration eliminates the errors encountered in trying to calibrate an instrument permanently in terms of temperature or osmolality.

We have chosen two body fluids of interest in biomedical research to illustrate the accuracy of the instrument. Accurate standards of NaCl at molar concentrations of from 0 to 1 and at molar intervals of 0.100 were used, as they are easily prepared. The values for the standards were corrected (*5*) to give molal concentrations, and these were corrected (*6*) to give the corresponding osmolal (*1*) concentrations. In this way the concentration of the unknown solution may be determined and expressed as a fraction of the ideal number of moles per kilogram of water (*1*). The results obtained from determinations on nine samples of human blood plasma are presented in Table 1. The values for the unknowns fell between those for the 0.1*M* (0.187-osmolar) and the 0.2*M* (0.360-osmolar) standards, and they were determined from the readings for the standards on the potentiometer dial. Determinations for standards and plasmas were made twice, and average values were computed. In Table 1, these values are compared with values obtained with a commercial macro-freezing-point apparatus (*7*) which has a nominal error of 2 percent or 1.0 millios-mole per kilogram of water, whichever is greater. Although several of the percentage errors in our determinations are high, the average error is 1.80 percent, a value which is within the accuracy of the control instrument. Table 2 shows the results obtained for nine samples of human urine. Except for the use of different standards, the method is identical with that used in making the determinations for plasma. The errors averaged 1.4 percent.

Table 2. Data from determinations on samples of human urine.

Sample No.	Micro method		Macro method
	Millios-moles/kg of H_2O	Error* (%)	Millios-moles/kg of H_2O
1	296	0.68	294
2	789	.50	793
3	427	.71	424
4	318	.31	319
5	223	3.72	215
6	361	2.85	351
7	364	0.83	361
8	407	.99	403
9	477	2.05	487

* Average error, 1.40.

The time required for determining the freezing points of eight samples (unknowns and standards) is now about 30 minutes. Sixty percent of this time is spent in loading the samples and flushing the pipet to prevent contamination. Once a standard procedure has been established by the technician and experience has been gained in controlling the thawing of the final crystal, the procedure should require less time.

Because of the quality of the results obtained with the instrument and the convenience and assurance with which determinations can be made, we feel that this method presents a practical, convenient means of determining freezing-point depression in millimicroliter samples.

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4. The assembly used was manufactured by Frigistors, Ltd., of Canada (model IFB-04-015-D1).
5. The corrections were based on data in C. D. Hodgman *et al., Handbook of Chemistry and Physics* (Chemical Rubber Publishing Co., Cleveland, 1949), p. 1636.
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12 August 1963