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Dehydration Studies On Bovine Spermatozoa

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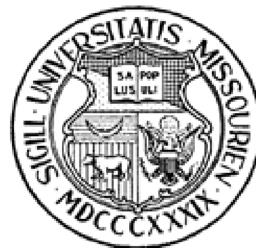

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SUMMARY

Several dehydration methods have been applied to bovine spermatozoa ± various diluents. These methods have included freeze-drying, solid-liquid extraction, liquid-gas extraction and solid-liquid-gas extraction. No motile spermatozoa were observed after dehydration by any of these methods and storage and subsequent reconstitution. Preliminary results with spermatozoa suspended in a glycerolated extender indicated that the potential for motility was maintained in the freeze-drying process until residual moisture content of the sample dropped below approximately 10 to 15 percent.

The freezing point curve for various concentrations of egg yolk-sodium citrate-glycerol diluent mixtures deviated markedly from the freezing point curve of corresponding glycerol-water solutions. The possibility of vitrification rather than freezing is indicated for some concentrations of the semen diluent mixtures.

A method has been described for estimating sublimation rates during the dehydration of bovine spermatozoa. Preliminary results indicate differences between bulls and/or ejaculates in sublimation characteristics of the semen.

CONTENTS

Introduction 4
Review of Literature 4
  Dehydration of Biological Materials 4
    Lyophilization or Freeze-Drying 4
    Solid-liquid Extraction 8
    Liquid-gas Extraction 8
  Application of Freeze-drying Technique to Spermatozoa 9
    Freeze-drying of Fowl Spermatozoa 9
    Freeze-drying of Bull, Ram and Rabbit Spermatozoa 10
    Freeze-drying of Human Semen 12
Materials and Methods 12
  Semen Collection 12
  Eutectic Point Determination 12
  Dehydration Methods 13
    Prefreezing Techniques 13
    Semen Freezing 13
    Moderate Vacuum Dehydration 14
    High Vacuum Dehydration 14
    Solid-liquid Displacement Dehydration 16
    Liquid-gas Displacement Dehydration 16
    Solid-liquid-gas Displacement Dehydration 16
  Rehydration Media 17
Observations and Results 17
Discussion 31
Bibliography 35

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Dehydration Studies On Bovine Spermatozoa

M. M. ANDERSON AND C. P. MERILAN

INTRODUCTION

The advent of frozen semen has opened up new possibilities for artificial breeding, but because the semen must be maintained at very cold temperatures, storage is expensive. Ice at −50° C is known to be almost as potent a drying agent as phosphorus pentoxide and since freezing is merely a process of removing liquid, a frozen spermatozoan probably can be considered dry, i.e., free of water at temperatures in the order of −79° C. If spermatozoa could be preserved in the dry state at room temperature, costs could almost certainly be lowered and artificial breeding could be extended into areas where it is not now economically feasible. Spermatozoa might also survive longer in the dried state than in the frozen. With these ideas in mind, and with the knowledge that bacteria may be dried and stored for years, experiments were conducted to test the applicability of certain dehydration methods for bovine spermatozoa.

REVIEW OF LITERATURE

Dehydration of Biological Materials

Lyophilization or Freeze-Drying:

The terms "lyophile" and "freeze-drying" were originally applied to serum dehydrated rapidly and then rapidly dried under vacuum (Flosdorf, 1949). In this usage, the colloid-chemial term "lyophile" (solvent-loving) is given a somewhat special meaning. The term is fitting, however, because it emphasizes an important characteristic of the dried material.

At atmospheric pressure of 760 mm. Hg., water boils at 100° C. Lowering the pressure above the water lowers this boiling point. This method, low temperature vacuum evaporation, has long been used for concentrating and drying solutions which would not be stable at higher temperatures. However, when drying delicate proteins some difficulties arise. As the solution concentrates, the

proteins come into contact with very high salt concentrations and denaturation of the molecules takes place. Although very stable proteins may survive this treatment, the more delicate ones are completely destroyed (Greaves, 1954).

If the pressure above the sample is reduced below the vapor pressure of ice, drying will occur from the frozen state. The ice sublimes and the water is removed from the solid mass without passing through a liquid phase. In the freeze-drying method the substance is frozen and the ice sublimed, leaving the protein and the salt molecules separate in a dry, spongelike structure. Since the substance is dried from the solid state, aggregation of molecules and concentration of salts cannot take place (Greaves, 1954). Such a process is capable of producing an extremely soluble product which does not appear denatured.

There are several possible reasons for drying by sublimation. According to Flosdorf (1949), for any given product one or more of the objectives below may be the reason for using this method:

1. Low temperature. In many cases a low temperature is necessary to prevent chemical changes during drying. This applies to blood plasma and serum, to most viruses, to many bacteria, and to various other biological and pharmaceutical materials.
2. Low volatility. Even though the sublimation is carried out under vacuum, the loss of volatile materials is said by Flosdorf to be small. This is because the decrease in vapor pressure with lower temperature is greater with many substances than with water.
3. No foaming. If dried at the proper temperature, there is no bubbling or foaming, and thus no harmful changes due to surface action.
4. Permanent dispersion. The dissolved substances in most cases remain evenly dispersed throughout the solvent during drying. The dry residue is a porous mass occupying about the same volume as the original solution. This aids the solubility of the dried product.
5. Minimal coagulation. As the solvent sublimes, the molecules of solute and colloidal particles stay in position, and little coagulation occurs.
6. No "case-hardening". Since there is no continual mixing or churning of solution but, rather, a gradual recession of ice, "case-hardening" does not occur.
7. Sterility. Bacterial growth and enzymatic actions cannot take place during drying. Furthermore, the final dried product resists bacterial growth.
8. No oxidation. Because of the high vacuum used in freeze-drying, there is not enough oxygen present to harm the sample.

Most biological products have an initial freezing point slightly below 0° C. However, although appearing solid, a sample may not be completely frozen. The presence of the unfrozen portion of the solution often becomes apparent by the release of bubbles after the material is placed under vacuum (Greaves, 1954). Flosdorf (1949) has called this condition "puffing." Also, a liquid phase may separate or the sample may completely melt. These undesirable conditions may

be prevented by drying at a temperature below the point at which all components of the sample are completely frozen, i.e., below the eutectic (Greaves, 1954).

The eutectic of mixtures of substances can be determined from phase diagrams constructed through the use of cooling curves. These phase diagrams show the temperatures and pressures at which a change in state occurs within the sample. Thus a mixture of ice, water, and water vapor consists of three phases (Daniels, 1952), but changes in temperature and/or pressure will favor the continued existence of only one or two of these phases. In using cooling curves to establish phase diagrams, a mixture of known composition is heated until it is completely melted. Then it is allowed to cool and the temperature is recorded frequently. By plotting time vs. temperature, a smooth cooling curve is obtained. However, whenever a solid phase separates, heat equal to the heat of fusion is evolved and offsets fully or partially the heat lost from the melted material and container. The rate of cooling decreases and the temperature at which an inflection is noted in the slope of the line can be used in plotting the phase diagram (Daniels, 1952).

Lane (1925) determined the phase diagram for glycerol-water in Figure 1 by this method of taking known compositions, noting the temperatures at which the solutions freeze, then plotting these temperatures vs. concentration and drawing the curve. This indicates a eutectic of –46.5° C for glycerol-water mixtures.

Although in theory drying should take place below the eutectic temperature of the solution, this is not always practical. Since the vapor pressure of ice becomes very low in extremely low temperature ranges, dehydration is slowed tremendously. For example, Flosdorf (1949) states that it would take about six weeks to dehydrate 20 grams of ordinary tissue at –65° C. Consequently, a compromise temperature is often used. A practical method of determining this temperature is to dry the substance at various temperatures, noting any tendency for bubbles to form on the surface, contraction in volume of the dried material, and "scaliness" of the dried material. Provided the eutectic mixture is small compared with the quantity of protein, the amount of denaturation of the product which occurs on drying will usually be slight (Greaves, 1954).

Before drying can take place from the solid state, it is necessary to freeze the solution. Since drying can take place only from a surface, the rate of drying must vary with the surface area from which evaporation can take place. This surface can often be regulated during the freezing process. The methods used in freezing will depend upon the substance to be dried, the two main methods being evaporative freezing (freezing by rapid evaporation under high vacuum) and pre-freezing (Greaves, 1954).

It is sometimes mistakenly thought that because lyophilization is carried out under high vacuum, less heat is required. Thermodynamically, the energy required to sublime a gram of ice at a given temperature to produce vapor, at

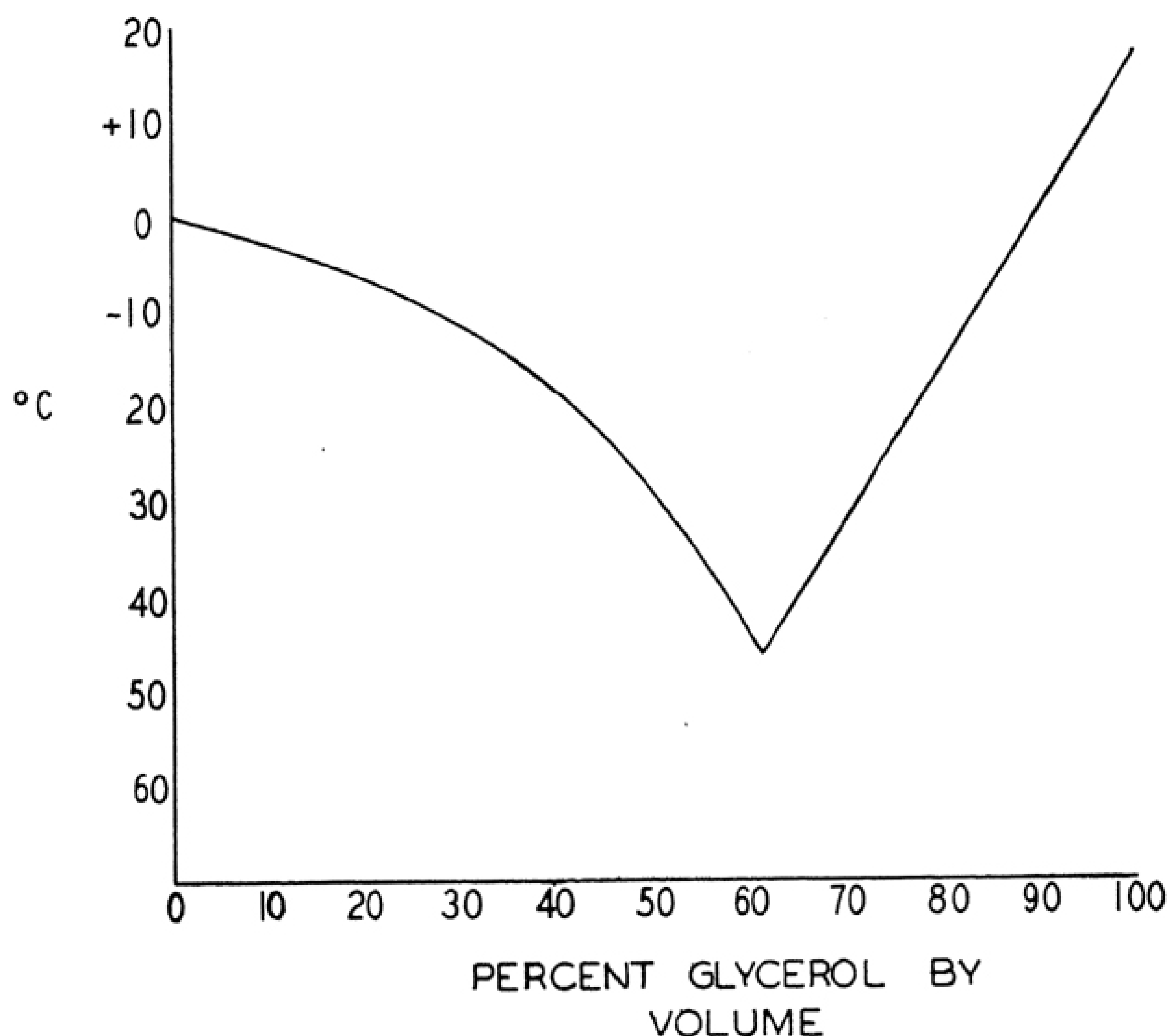

Fig. 1—Freezing points of glycerol-water solutions.

room temperature, for example, is equal to the heat of fusion of ice and the heat of vaporization of water at room temperature plus the heat necessary to raise the temperature of the ice to the freezing point and the water to room temperature. The thermodynamic relations hold whether the process is carried out slowly at atmospheric pressure or rapidly under vacuum (Flosdorf, 1949).

Thus, the rate of drying is closely related to the amount of heat applied to the sample. How quickly heat may be applied will depend upon the rate of heat transfer through the material and the highest safe temperature at which the substance may be dried.

During the final stage of drying, the product is usually taken to as high a temperature as it will stand in order to reach a minimum moisture content in the shortest possible time. The final temperature varies. Some products, for example, viruses and many living bacteria, cannot be taken much above room temperature, while blood plasma may be taken as high as 80° C (Flosdorf, 1949).

The applications of freeze-drying or lyophilization for the preservation of biological materials have been numerous. They include drying medical products (blood serum and plasma, bacterial and viral cultures and vaccines, antibiotics, hormones, vitamins, tissues, and others), food products, and industrial products (Flosdorf, 1949).

Solid-liquid Extraction:

According to Mueller and Szent-Gyorgyi (1957), acetone at dry ice temperature does not denature proteins but still is capable of dissolving out ice to some extent. Also, chlorated paraffins do not harm contractile proteins at room temperature. Knowledge of these facts enabled them to dehydrate muscle without destroying its molecular structure and contractility.

Fresh frog sartorius muscles or thin strips of fresh rabbit psoas were immersed in anhydrous acetone at dry ice temperature and stored for a week. The acetone slowly dehydrated the frozen muscle. The fluid was then replaced with fresh precooled acetone and the muscle left in this liquid for two weeks, or, instead of exchanging the acetone, the water extracted from the muscle was bound with calcium chloride. After dehydration, the muscle was transferred into precooled ethyl chloride and left for a week or so at dry ice temperature in order for the ethyl chloride to extract the acetone. Next, the muscle was taken out of the solvent and warmed to room temperature. The white, lightweight preparation obtained had the general appearance and shape of the original muscle. Upon immersion into water, it contracted violently to one-third to one-fifth of its origiginal length. The dried muscle could be kept for several weeks in a desiccator without loss of contractility, but when left in an open vessel, it lost that ability within a few days.

To date, this method of dehydration has not been widely used for biological materials.

Liquid-gas Extraction:

Kistler (1932) developed a method for removing the liquid phase from gels by taking advantage of the point well known to physical chemists as the "critical temperature." The critical temperature is that temperature above which a gas cannot be liquefied by pressure alone (Hodgman, 1949). Therefore, if a liquid is held under a pressure always greater than the vapor pressure and heat is applied, the liquid will be transformed at the critical temperature into a gas without two distinct phases having been present at any time. Actually, according to Kistler, the change that does take place is continuous and gradual over the entire thermal range and a small increase in temperature from slightly below the critical point to slightly above has no more meaning to a gel in the liquid than a similar change in temperature at any other place. Kistler's procedure was simply to take a gel filled with liquid, transform the liquid gradually into a gas by

warming it above the critical temperature, allow the gas to expand, and then have remaining a gel filled with gas of low density. During this process the gel is never subjected to the compressive forces usually associated with drying.

Kistler (1932) first attemped to dry inorganic jellies filled with water simply by taking the water above its critical point and releasing it as a vapor. This was difficult because water has inconveniently high critical points. But more serious than this, as the critical temperature was approached water was found to become such a powerful peptizer and solvent that gels such as silica or alumina were completely peptized. This difficulty was conquered by first replacing the water with another liquid or series of liquids before drying. For example, the organic gels were usually transferred from alcohol to ether, to petroleum ether, and finally to liquid propane. By removing the water from normal gels, Kistler successfully formed aerogels of silica, alumina, tungistic oxide, ferric oxide, stannic oxide, nickel tartrate, cellulose, nitrocellulose, gelatin, agar, and egg albumin.

Anderson (1950, 1951) prepared specimens for the electron microscope by Kistler's method. After fixing the sample with osmic acid vapor, he replaced the water with absolute alcohol by taking the sample through increasing concentrations. The alcohol was replaced by amyl acetate and the amyl acetate by liquid carbon dioxide. The carbon dioxide was then raised above its critical point, an escape valve opened slightly, and the gas allowed to evolve. Using this method, Anderson removed water from biological systems while preserving many aspects of their three dimensional structures. Although he achieved better results than by air drying or freeze-drying, some distortions still occurred.

Application of Freeze-drying Technique to Spermatozoa

The literature pertaining to attempts at freeze-drying spermatozoa is relatively meager. However, attempts have been reported at drying semen of the fowl, bovine, ram, and human.

Freeze-drying of Fowl Spermatozoa:

Freeze-drying of semen was first reported by Polge, Smith, and Parkes (1949). Working with semen of fowl they were able to obtain up to 50 percent motile sperm after drying and reconstitution of the apparently dry material. One cc. of fowl semen was diluted with 1 cc. of 20 percent glycerol in Ringer's solution, cooled to −79° C, and allowed to warm to −25° C. The flask was then connected to a high vacuum distillation system. After three hours, at which time the distillation was stopped, the semen had the appearance of being dry and 1.7 cc. of water was thawed from the condenser. While still cold, the dehydrated semen was reconstituted with 1.8 cc. of water and warmed to 40° C. Upon microscopic examination with a 4 mm. objective, active spermatozoa were seen in each field. The authors state that in subsequent experiments using 30

percent glycerol in Ringer's solution, about 90 percent of the water was removed and up to 50 percent motile spermatozoa were observed.

Freeze-drying of Bull, Ram, and Rabbit Spermatozoa:

Sherman (1957) attempted to preserve bull semen by lyophilization. After preparation with an egg yolk-glycerol extender in the normal way for freezing, smears of each sample of diluted semen were then spread on four glass microscope slides. The semen was frozen in a closed metal container by placing the container with the slides in an insulated box at -70° C. After 30 minutes two slides were transferred to the refrigerator chamber (-65° C) of the freeze-dry apparatus while the other two slides were left at -70° C to serve as controls. Semen smears of each sample were subjected to vacuum desiccation at this low temperature for periods ranging from 6 to 48 hours, after which they were reconstituted by adding either one drop of distilled water or one drop of sterile Lock's solution to each of the slides. Sherman states the results quite concisely, "Not one motile spermatozoon was observed subsequent to exposure to this process for 6 hours or more."

Bialy and Smith (1957) also attempted to dry and reconstitute bull spermatozoa. An egg yolk-citrate extender consisting of 23 percent egg yolk by volume and 2.23 percent sodium citrate dihydrate was used as the diluent. Then the diluted semen was mixed with an equal volume of diluter containing 15 percent glycerol, giving a final glycerol content of 7.5 percent. After an equilibration time of 14 to 18 hours at 4° to 6° C, the semen was frozen as follows: 4° C to -10° C, one degree every three minutes; -10° C to -15° C, one degree per minute. The temperature was then lowered rapidly and -70° C to -75° C was reached within 6 to 10 minutes. The frozen material was attached to a freeze-dry apparatus and maintained at -65° C to -70° C until the pressure reached about 250 microns, at which time the semen container was immersed into a Dewar flask filled with alcohol at the desired temperature. A drying temperature of -70° C and various temperatures from +20° C to -35° C were used. Rehydration was accomplished by adding cold distilled water, 0° C to + 5° C, to the dehydrated samples in amounts equivalent to the weight lost.

When the drying temperatures in the work of Bialy and Smith (1957) were varied from -35° C to 20° C, no obvious pattern in the relation of the drying temperature to the survival rate could be observed, and the data were grouped according to degree of dehydration. In one trial a few motile cells were observed when the dehydration was determined to be from 90 to 95 percent. However, the thickness of the semen layer was considerable and, under these conditions, uniform drying cannot be expected unless dehydration is complete. As there were no stored dried samples, the question concerning live dehydration ranges remains unanswered. Cytological evidence shows that freeze-drying at temperatures below the eutectic point should produce few cytophysiological changes.

The drying temperature of –70° C should fulfill this requirement. However, in these experiments freeze-drying at –70° C followed by rehydration in the normal manner yielded negative results whenever the dehydration level exceeded 71 percent.

Leidl (1956), also working with egg yolk-sodium citrate diluted bull semen, likewise found that in some instances motile spermatozoa could be seen after freeze-drying, provided the final water content did not go lower than about 5 percent. Leidl's procedure differed somewhat in that he began drying at a temperature of -79° C. and then allowed the temperature to rise gradually over a period of four to five days.

Albright, *et al.* (1958), using a whole milk-glycerol extender, cooled bull semen in a dry ice bath and began freeze-drying as the temperature dropped below about -50° C. After a 45 minute lyophilization period and subsequent period during which the samples were held at dry ice temperature, the spermatozoa were reconstituted by the addition of a 3 percent fructose solution at 37.8° C. The authors state that approximately 5 to 10 percent of the spermatozoa showed progressive motality. However, they made no attempt to store the semen at relatively warm temperatures or to determine the amount of water removed.

A Russian worker, Yushchenko (1957), has reported success in the freeze-drying of bull, ram, and rabbit spermatozoa. This report indicated successful storage of dried bull spermatozoa for 18 to 20 months at room temperature, after which 15 to 20 percent of the spermatozoa could be revived. Live spermatozoa reportedly were observed after keeping ram and rabbit spermatozoa in the dry state at 18° C. to 20° C. for six to eight months. Rehydration was either by use of distilled water or sodium citrate solutions or other normal dilution media. Fertility of dried rabbit spermatozoa was checked by inseminating female rabbits, and the report states that 12 litters of normally developed rabbits were obtained, ranging from two to eight young per litter.

After examination of fresh semen, Yushchenko (1957) diluted it with a synthetic medium, cooled it for 3 to 4 hours, then diluted it with a medium containing 7.5 to 20 percent glycerol. In a separate container, freons 113, 114, and 11 were mixed with n-heptane in such a manner as to make a fluid with a specific gravity of 1.18-1.44. After the semen had equilibrated with glycerol for 12 to 24 hours, it was poured into test tubes containing the mixture of freons and heptane. The spermatozoa were then forced down into the freons-heptane mixture by centrifugation at 6000 to 7000 rpm for 15 to 30 minutes. The spermatozoa, after being frozen to a temperature of between -20° C. and -78° C., were dried under a vacuum of 10^{-3} to 10^{-4} mm. Hg. Dry ice-alcohol or liquid nitrogen condensers were used in the vacuum system. The report indicates that best results were obtained with bull semen by drying in the frozen state at –2° C. to –3° C. for 2 hours. Yushchenko stated that results were significantly better in the experiments conducted in the preceding manner than in those where spermatozoa were frozen and dried in the normal water-containing medium.

More recently, Meryman (1959) has reported conception in a cow following insemination with dehydrated semen. The semen had been reconstituted without an intervening storage period following freezing and dehydration by vacuum sublimation. The residual moisture content of the spermatozoa was not determined but was probably less than 8 percent. In his report Meryman emphasized the fact that the majority of the spermatozoa died when stored over silica gel for 24 hours or longer. Although the dehydration and/or storage techniques did not provide the "stabilized" conditions necessary for storage in the dry state, the experiment provided needed evidence of viability and fertility in spermatozoa from which the major portion of water had been removed.

Freeze-drying of Human Semen:

Sherman (1954), in an earlier study, performed essentially the same experiments on human semen as the work he reported on bull spermatozoa. One part of glycerol was mixed with nine part of liquefied semen, frozen on slides at four different rates and dried for periods ranging from 6 to 48 hours. Reconstitution was as in the case of the bull semen and the results were also negative.

MATERIALS AND METHODS

Semen Collection

Bovine semen was collected by means of an artificial vagina from healthy bulls in the University of Missouri Agricultural Experiment Station dairy herd. The semen was protected against cold shock and transported to the laboratory where it was diluted as desired and cooled gradually. Motility ratings were made using a 0-5 scale as described by Herman and Madden (1953). A (+) or (-) was sometimes used to indicate small differences between samples.

Eutectic Point Determination

Samples were prepared by adding glycerol in various quantities to a diluter prepared as directed by Swanson (1954) and consisting of 20 percent egg yolk by volume and 80 percent by weight of a 3 percent solution of sodium citrate dihydrate.

The sample, in a double-wall test tube, was surrounded by an alcohol-glycerol solution in a metal container. This container, in turn, was surrounded by an alcohol-dry ice bath. When temperatures colder than -60° C. were desired, liquid air was used as the coolant. Sample temperature as measured by copper vs. constantan thermocouples or thermopiles referenced against ice water at 0° C. was recorded as the corresponding voltage by a Minneapolis Honeywell Brown Electronik multipoint 2 second interval recording potentiometer. Freezing of each sample was indicated by an abrupt change in the rate of cooling. This resulted from the evolution of heat equal to the heat of fusion. The temperature at which

this happened was plotted vs. the glycerol concentration of the sample to give a point on the phase diagram.

Dehydration Methods

Prefreezing Techniques:

The diluents used for extending semen were:

1) M/10 potassium citrate solution.
2) 20 percent egg yolk (by volume).
   80 percent M/10 sodium citrate solution (by volume)
3) 20 percent egg yolk (by volume).
   80 percent M/10 potassium citrate solution (by volume).
4) Skim milk prepared by heating to 95° C. and holding for 10 minutes.
5) 20 percent egg yolk (by volume).
   80 percent (by volume) skim milk prepared by heating to 95° C. and holding for 10 minutes.

Prior to freezing, semen in each of the described diluents was further extended by the addition of an equal quantity of the same diluter containing 16 percent glycerol. The resultant 8 percent glycerol mixture was left to equilibrate for 6 to 24 hours at refrigerator temperature. In several trials, glycerol was replaced by methyl alcohol or partially replaced by propylene glycol. Dilution ranged from 1 part semen plus 3 parts diluter to 1 part semen plus 20 parts of diluter.

Centrifugation of equilibrated spermatozoa for 10 minutes at 2000 rpm (100 rpm = 30 x gravity) and subsequent removal of the supernatant liquid was employed in certain trials to decrease the amounts of glycerol and water present in the sample.

In experiments similar to those reported by Yushchenko (1957), various percentages of freons 113, 114, and 11 were mixed with Eastman Kodak Chemical P2218 (50 percent n-heptane, 30 percent methylcyclohexane, 15 percent isoheptane). Spermatozoa were diluted, equilibrated with glycerol for 6 to 24 hours, and transferred into test tubes containing the freons and Eastman Kodak Chemical. The spermatozoa were then centrifuged in a Servall Superspeed Type SS-1 Angle Centrifuge at speeds ranging from 7000 to 15,500 rpm (15,500 rpm = 31,000 x gravity) for 5 to 30 minutes. The water-containing medium above the organic mixture was removed with a suction pipette and the organic liquid containing the spermatozoa was then placed in glass dehydration tubes, frozen, and attached to the lyophilizer.

Semen Freezing:

Diluted semen, after 6 to 24 hours glycerol equilibration, was frozen by decreasing the temperature 1° C. per minute to -20° C., then cooling more rapidly to −79° C. In some trials, 0.1 ml. semen was "shell-frozen" around the inside of

approximately 8 mm. glass bulbs in order to increase the surface area and drying rate. When freezing spermatozoa in the freons-Eastman Kodak Chemical P2218 mixtures, several different cooling rates were used. In certain experiments, propylene glycol was substituted for half the glycerol; in others, the glycerol was omitted and methyl alcohol was used (3 percent final concentration). In the latter case, only a 20 minute equilibration period was given.

Moderate Vacuum Dehydration:

A Stokes 102-L cryochem apparatus with a Cenco Presso-Vac Pump and McLeod vacuum gauge were used. Water and any organic vapors which sublimed were trapped by a chemical condenser containing calcium sulfate and a small amount of silica gel. Sample tubes were either 7 mm. or 11 mm. (outside diameter). However, the 11 mm. tubes were constricted to 8 mm. toward the top.

Temperature control of the samples during dehydration was accomplished by an insulated fluid bath surrounding the sample tubes. This bath was generally maintained at -79° C., -20° C., -15° C., -10° C., or -3° C. However, in a limited number of trials, lyophilization was begun at -79° C. and the sample allowed to warm slowly to room temperature.

Oxidants such as potassium permanganate, potassium or sodium dichromate, and chromic acid have been found to oxidize glycerol to numerous products, including carbon dioxide and water, glyceraldehyde, and acetone. The quantity of each product is determined by the pH of the solution. Glycerol and potassium permaganate crystals react so violently that they may even ignite (Segur, 1953). In an attempt to oxidize the glycerol vapor in the system and thus aid vaporization of glycerol from the sample, semen was dried with potassium permanganate crystals between the samples and the lyophilizer. All vapors removed thus passed over the crystals.

High Vacuum Dehydration:

A Cenco Presso-Vac forepump lowered the pressure in the apparatus as diagramed in Figure 2 to less than 100 microns Hg. The mercury diffusion pump further reduced the pressure to less than 0.001 micron. A McLeod gauge enabled estimation of vacuum from about 370 microns to about 0.02 micron and also made it possible to judge when the pressure was below approximately 0.001 micron Hg. As the vapors sublimed from the 28 mm. (outside diameter) flat-bottom sample tube, they condensed in a liquid air or dry ice cold trap. The bath temperature surrounding the sample was held at -20° C., -10° C., or -3° C., or drying was started at -79° C. or -40° C. and the sample allowed to warm slowly.

In obtaining sublimation curves, temperature difference between samples and bath was determined by copper:constantan thermocouples attached to a Kintel Model 204A electronic galvanometer and amplifier. The amplified electro-

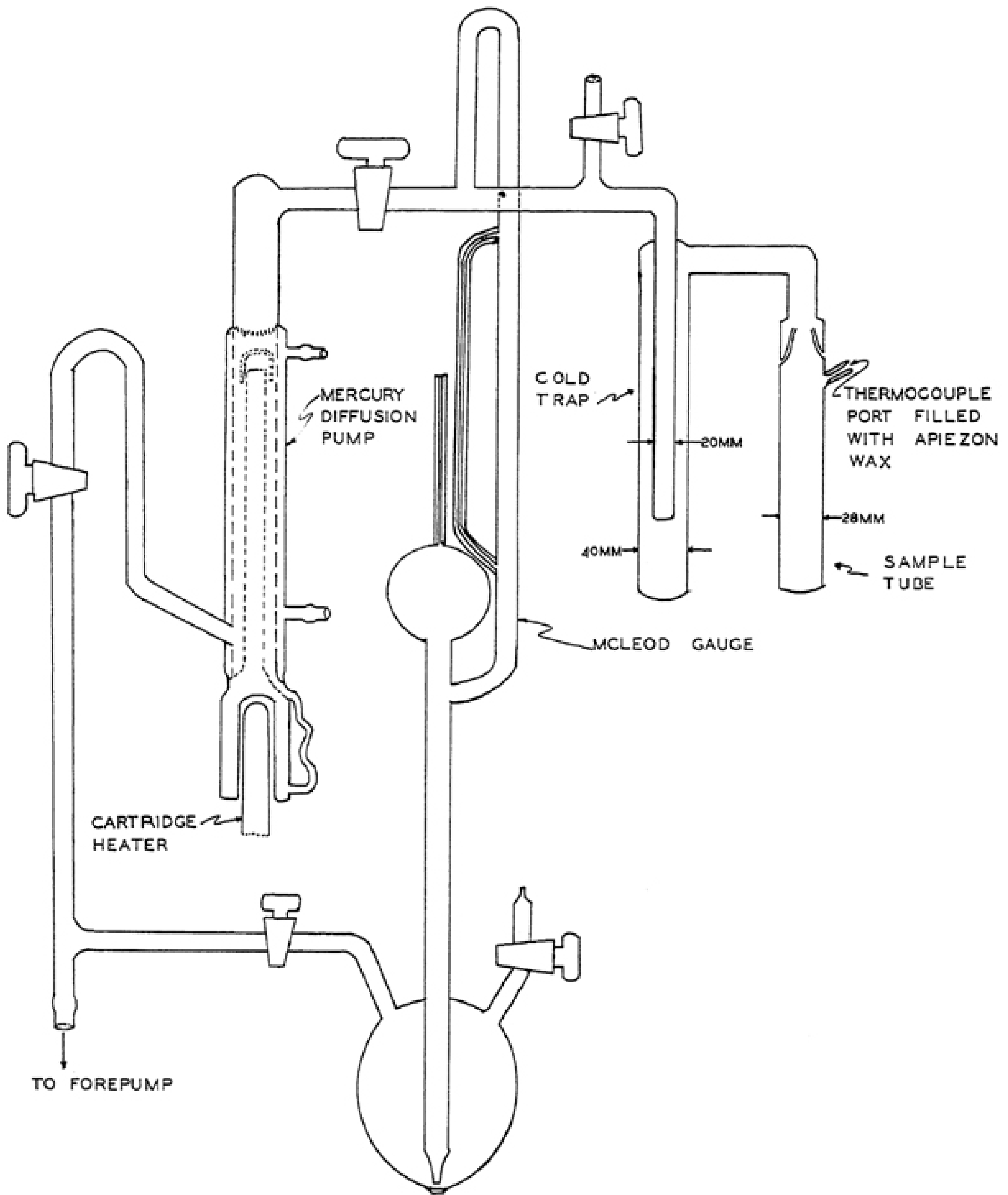

Fig. 2—High vacuum apparatus.

motive force was then recorded by a 0-10 mv. Minneapolis Honeywell Brown Electronik recording potentiometer. The measuring junction, positioned 1 mm. above the bottom of the sample tube, was referenced against bath temperature at the outside of the tube. With the thermocouple in place, 1.5 ml. of semen was frozen in the bottom of the tube. The sample tube containing frozen semen

at dry ice temperature was attached to the apparatus, placed under vacuum, and allowed to warm slowly. When a substance sublimed, heat was absorbed, the measuring junction was cooled, and an electromotive force corresponding to the temperature difference between junctions was created in the thermocouple. Bath temperature was measured with a thermometer.

Solid-liquid Displacement Dehydration:

Semen prepared and frozen in the previously indicated manner either on slides or in volume (which was mechanically broken into bits) was immersed in an organic liquid at -79° C. The liquid slowly dissolved ice from the sample. Each 2 weeks the liquid was replaced. When drying was considered complete, the fluid was removed from the semen either by evaporation at storage temperatures or by rapid evaporation under vacuum at warmer temperatures.

Liquid-gas Displacement Dehydration:

A liquefied gas under pressure was used to replace the water in spermatozoa. The sample was then warmed above the critical temperature of the gas, the pressure slowly released, and the gas allowed to evolve. Resistance of spermatozoa to pressure was determined by exposing semen to helium or nitrogen in a combustion bomb. The following gases have critical temperatures in a feasible range for dehydration by this method, i.e., from about 0° C. to about 38° C.: acetylene, carbon dioxide, ethane, ethylene, xenon, and nitrous oxide (Hodgman, 1949). Relative toxicity of carbon dioxide, ethane, and ethylene was determined by placing semen in a 3-ml. brass chamber and admitting each gas under various pressures, temperatures, and for various periods of time, so that it came into contact with semen both as a gas and as a liquid. Admission or release of gas was kept slow to minimize harmful temperature changes. When attempting dehydration, semen was placed in a 3-ml. brass chamber; gas was admitted and liquefied; the chamber was inverted occasionally; the sample was warmed above the critical temperature of the gas; and the gas released.

Solid-liquid-gas Displacement Dehydration:

In this technique, solid-liquid displacement followed by liquid-gas displacement, semen was diluted with egg yolk-sodium citrate-glycerol and frozen. The solid mass was broken up by physical means and small pieces were dropped into a beaker of acetone at -79° C. Each two weeks the liquid was replaced with fresh precooled acetone. After exposure for about six weeks, a piece of the frozen material was removed, placed in a 3-ml. brass pressure chamber, and liquid ethane at -79° C. was admitted. The chamber was inverted occasionally during a 10-minute period; then the pressure cell was warmed in a water bath at 38° C. An escape valve was opened slightly and the gas was released.

Rehydration Media

Rehydration of dried or partially dried spermatozoa involved the addition of liquid at either + 5 C. or +25 C. Liquids or solutions used were distilled water, 3 percent sodium citrate dihydrate, 3 percent potassium citrate monohydrate, Beckman 3501 pH 7 buffer, egg yolk-sodium citrate diluter, aged bacterial contaminated egg yolk-sodium diluter, skim milk, egg yolk-skim milk diluter, and solutions of adenosine triphosphate.

OBSERVATIONS AND RESULTS

Figure 3 shows the results of freezing point determinations with various glycerol-egg yolk-sodium citrate mixtures. With 50 percent glycerol by volume, freezing occurred at -55° C. No freezing or thawing was detected with higher glycerol concentrations even though samples were cooled as low as -145° C. and warmed slowly. Viscosity appeared to pregressively increase upon cooling until about -60° C. was reached, but no change was noted at lower temperatures.

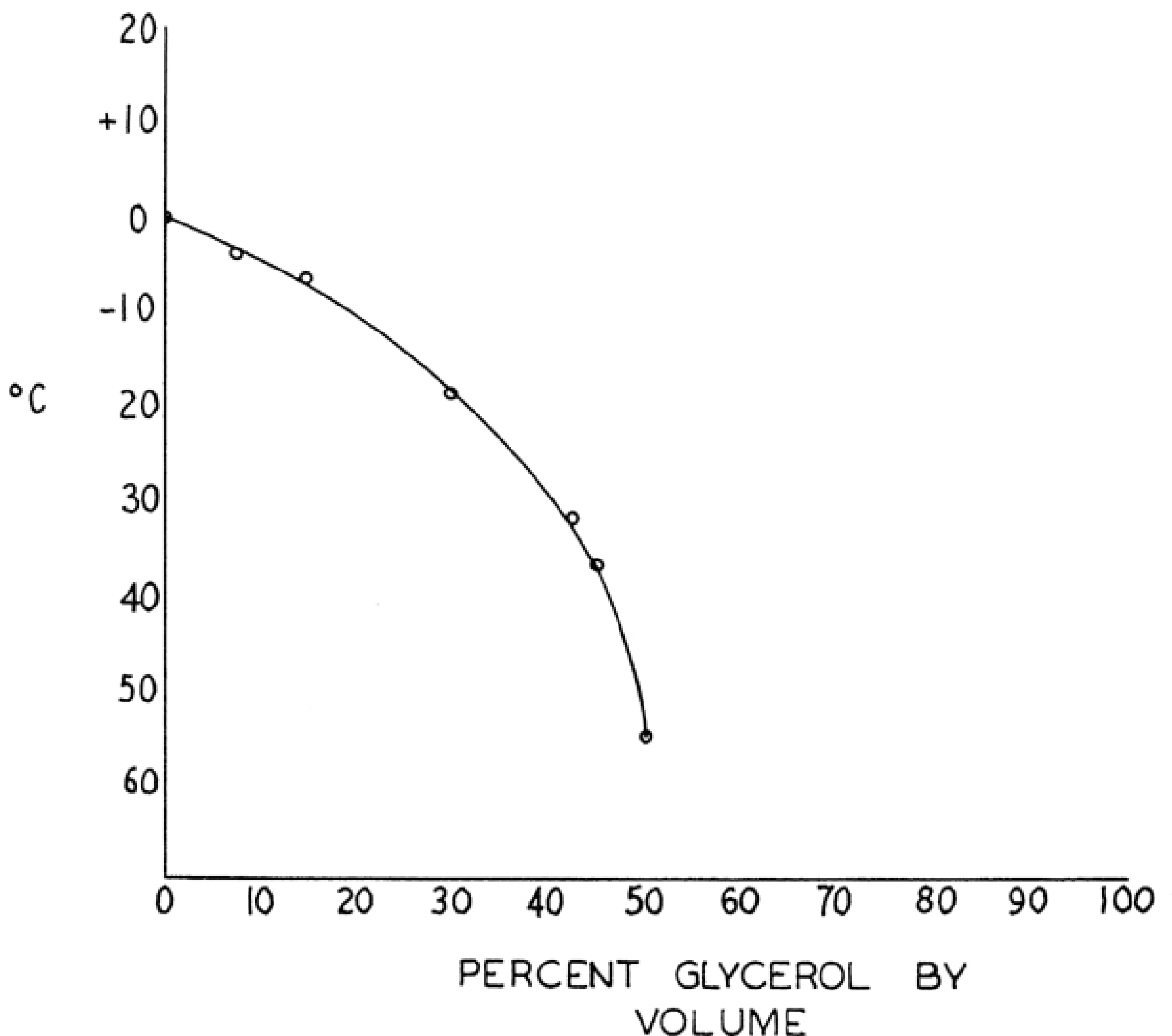

Fig. 3—Freezing points of glycerol-egg yolk-sodium citrate solutions.

Post-freezing motility observations indicated that methyl alcohol (3 percent final concentration) afforded spermatozoa fairly good protection against freezing at temperatures warmer than about -20° C. It was also found that a final concentration of 4 percent glycerol and 4 percent propylene glycol gave reasonably good protection to egg yolk-sodium citrate diluted spermatozoa at temperatures warmer than about -45° C.

Centrifugation of diluted, glycerol equilibrated semen for 10 minutes at 2000 rpm (100 rpm = 30 x gravity) packed the cells loosely at the bottom of the centrifuge tubes. After removal of the supernatant liquid and subsequent freezing of the cells, motility was found to be slightly lower than in non-centrifuged controls.

"Shell-freezing" of 0.1 ml. diluted, glycerol equilibrated semen inside 8-mm. glass bulbs resulted in a thin, fairly even semen layer. Spermatozoa motility was slightly lower than in identical semen samples frozen in bulk.

In experiments similar to those reported by Yushchenko (1957), spermatozoa were equilibrated with egg-yolk-sodium citrate-glycerol and with egg yolk-skim milk-glycerol. Final glycerol concentration was 8 percent in each case. One-tenth to 0.5 ml. of the equilibrated spermatozoa were then added to 3 to 7 ml. of mixtures of freon(s) and heptane (practical). The freons used were 11, 113, and 114. The heptane (practical) was Eastman Kodak Chemical P2218 (50 percent n-heptane, 30 percent methylcyclohexane, 15 percent isoheptane). Centrifugation for up to 30 minutes at 15,500 rpm (15,500 rpm = 31,000 x gravity) did not force either fresh or six-day old spermatozoa down into the organic mixture until the freons were reduced in specific gravity to about 1.15. At specific gravities higher than this, the diluter remained above the organic mixture and the spermatozoa packed in a thin layer along the diluter-organic liquids interface. Addition of Tween 80 (0.3 percent final concentration) did not cause the spermatozoa to centrifuge down into a 1.25 specific gravity mixture.

A specific gravity of about 1.15 was given by a mixture of 3 parts freon(s) and 4 parts heptane (practical). When this mixture was used, centrifugation for 5 minutes at 7000 rpm (15,500 rpm = 31,000 x gravity) massed the spermatozoa in a small clump near the bottom of the organic liquid mixture. When the organic liquids were poured off and 3 percent sodium citrate dihydrate quickly added, the spermatozoa appeared normal and showed only a slightly diminished motility. Spermatozoa motility after centrifugation did not appear dependent upon the type of freon(s) used. The supernatant liquid was removed with a suction pipette and the spermatozoa were frozen in the organic liquids. In most of the trials no survival was seen. However, after using 1 part of each of the freons (11, 113 and 114) and 4 parts of heptane (practical), up to about 5 percent of the spermatozoa were motile after freezing.

The lowest pressure reached in the Stokes 102-L lyophilizer was approximately 75 microns Hg., with the average pressure about 200 microns Hg. Table 1 gives general conditions of the moderate vacuum dehydration experiments.

TABLE 1-MODERATE VACUUM DEHYDRATION TRIALS

Diluent and Special Techniques	Drying Temperature	Rehydration Media	Remarks
1) Egg yolk-sodium citrate, 8% glycerol 2) Egg yolk-potassium citrate, 8% glycerol 3) Egg yolk-potassium citrate, 8% glycerol, $KMnO_4$ trap	-79° C.		Not dry after 12 days. Spermatozoa decreased slightly in motility.
1) Centrifuged 10 minutes at 2000 rpm (100 rpm = 30 x gravity): a) Egg yolk-sodium citrate, 8% glycerol b) Egg yolk-potassium citrate, 8% glycerol	-79° C.		Not dry in 7 days. Some motility remained.
1) Egg yolk-sodium citrate, 8% glycerol 2) Egg yolk-potassium citrate, 8% glycerol 3) Egg yolk-sodium citrate, 8% glycerol, $KMnO_4$ trap 4) Egg yolk-potassium citrate, 8% glycerol, $KMnO_4$ trap	Drying begun at -79° C. and temperature allowed to rise slowly	Water, sodium citrate, potassium citrate, Beckman 3505 buffer, egg yolk-sodium citrate, egg yolk-potassium citrate, sodium citrate + ATP, potassium citrate + ATP	No motile spermatozoa seen after samples reached apparent dryness.
1) Egg yolk-sodium citrate, 8% glycerol 2) Egg yolk-potassium citrate, 8% glycerol	-20° C. -10° C.	Water, sodium citrate, potassium citrate, Beckman 3505 buffer, egg yolk-sodium citrate, egg yolk-potassium citrate, sodium citrate + ATP, potassium citrate + ATP	No motile spermatozoa seen after samples reached apparent dryness.
1) Centrifuged 10 minutes at 2000 rpm (100 rpm = 30 x gravity): a) Egg yolk-sodium citrate, 8% glycerol b) Egg yolk-potassium citrate, 8% glycerol	Drying begun at -79° C. and temperature allowed to rise slowly	Water, sodium citrate, potassium citrate	Same as above

TABLE 1 CONTINUED

Diluent and Special Techniques	Drying Temperature	Rehydration Media	Remarks
1) "shell-frozen" in 8 mm. glass bulbs: a) Egg yolk-sodium citrate, 8% glycerol b) Egg yolk-potassium citrate, 8% glycerol	-20° C	Water, sodium citrate, potassium citrate	Same as above
1) Skim milk, 8% glycerol	-20° C.	Water, skim milk, sodium citrate	Same as above
1) Egg yolk-sodium citrate, 3% methyl alcohol	-15° C.	Water, sodium citrate, egg yolk-sodium citrate, aged egg yolk-sodium citrate	Same as above
1) Centrifuged 5 minutes at 7000 rpm (15,500 rpm = 33,000 x gravity) into 1 part freon 11, 1 part freon 113, 1 part freon 114, 4 parts heptane (practical) Eastman Kodak Chemical P2218 consisting of 50% n-heptane, 30% methylcyclohexane, 15% isoheptane): a) Egg yolk-sodium citrate, 8% glycerol b) Egg yolk-skimmilk, 8% glycerol	-20° C. - 3° C.	Water, egg yolk-sodium citrate, egg yolk-skim milk glycerol, sodium citrate	No motile spermatozoa seen after samples reached apparent dryness.

Only single lyophilization attempts were made at a constant temperature of –79° C. and dehydration was not complete. At least four separate trials were made under each of the other conditions indicated. After freeze-drying at temperatures warmer than –79° C., all samples seemed normal except that samples containing egg yolk lightened considerably in color. In general, the mass dissolved readily upon rehydration and the spermatozoa appeared morphologically normal. However, after having been dried, no motile spermatozoa were observed either at the time of rehydration or after 24 hours in the rehydrating fluids.

Table 2 gives the types of high vacuum dehydration experiments which were conducted. Pressures involved in these trials were typically less than 0.001 micron Hg. Upon drying, the samples containing egg yolk lightened in color. The raw samples and those diluted with potassium citrate or potassium citrate plus glycerol were glossy and contained small cracks. The spermatozoa appeared morphologically normal. However, upon rehydration and storage of the semen in the rehydrating fluids for up to 24 hours at refrigerator temperature, no motility was observed.

The sublimation curves given in Figures 4 to 9 were obtained by recording the apparent temperature difference between the sample in vacuo and the bath surrounding the sample. This temperature difference was expressed as the voltage generated by copper:constantan thermocouples. Three raw semen samples were dried (Figures 4, 5, and 6). In the first two, lyophilization was continued until the semen had reached apparent dryness. However, in the other experiment (Figure 6), dehydration was not complete. Figures 7 and 8 show the results of drying semen which had been diluted with equal parts of 0.1 molar potassium citrate before freezing. Figure 7 is for semen which was taken to dryness, but because of air leaks in the vacuum system, the other trial (Figure 8) was terminated before the semen was completely dry. Semen diluted with equal parts of a potassium citrate-glycerol mixture (8 percent final glycerol concentration) was also dried (Figure 9). The semen used was two days old except for the glycerol-containing sample, which was one day old. Post-freezing motility determinations on control samples showed motile spermatozoa only in the sample containing glycerol. The dried samples had a glossy appearance and contained several small cracks. Upon rehydration with potassium citrate, the spermatozoa appeared morphologically normal. However, no motility was observed.

In solid-liquid extraction dehydration trials, frozen egg yolk-sodium citrate diluted semen containing 8 percent glycerol was found to withstand seven days exposure to acetone at –79° C. with no noticeable decrease in spermatozoon motility. Semen smears frozen on glass slides required four to six weeks to reach apparent dryness, while small masses of semen required somewhat longer. The acetone was removed by evaporation at storage temperatures or by evaporation under vacuum at warmer temperatures. The dried sample was white in appearance. The mass did not rehydrate readily with water, sodium citrate buffer, or egg yolk-sodium citrate diluter. Many dark clumps were noted with the micro-

TABLE 2-HIGH VACUUM DEHYDRATION TRIALS

Diluent and Special Techniques	Drying Temperature	Rehydration Media	Remarks
1) Egg yolk-sodium citrate, 8% glycerol	Drying begun at $-79^{o}C$. and temperature allowed to rise slowly	Water, potassium citrate, sodium citrate, egg yolk-sodium citrate aged egg yolk-sodium citrate	Spermatozoa with no glycerol dead upon freezing. In other trials no motile spermatozoa seen after samples reached apparent dryness.
2) Egg yolk-potassium citrate, 8% glycerol			
3) Egg yolk-skim milk, 8% glycerol frozen on wood splints			
4) Egg yolk-skim milk, 8% glycerol			
5) Potassium citrate, 8% glycerol			
6) Potassium citrate			
7) Undiluted Semen			
8) Centrifuged 5 minutes at 7000 rpm (15,500 rpm=33,000 x gravity) into 1 part freon 11, 1 part freon 113, 1 part freon 114, 4 parts heptane (practical) Eastman Kodak Chemical P2218 consisting of 50% n-heptane, 30% methylcyclohexane, 15% isoheptane)			
a) Egg yolk-sodium citrate, 8% glycerol			
b) Egg yolk-skim milk, 8% glycerol			

TABLE 2 CONTINUED

Diluent and Special Techniques	Drying Temperature	Rehydration Media	Remarks
1) Centrifuged 5 minutes at 7000 rpm (15,500 rpm=33,000 x gravity) into 1 part freon 11, 1 part freon 113, 1 part freon 114, 4 parts heptane (practical) Eastman Kodak Chemical P2218 consisting of 50% n-heptane, 30% methylcyclohexane, 15% isoheptane):	-20°C. -10°C. -3°C.	Water, potassium citrate, sodium citrate, egg yolk-sodium citrate, aged egg yolk-sodium citrate	No motile spermatozoa seen after samples reached apparent dryness.
a) Egg yolk-sodium citrate, 8% glycerol			
b) Egg yolk-skim milk, 8% glycerol			
1) Egg yolk-sodium citrate, 4% glycerol 4% propylene glycol	Drying begun at -40°C. and temperature allowed to rise slowly	Water, sodium citrate, skim milk	Same as above
2) Egg yolk-skim milk, 4% glycerol, 4% propylene glycol			

Fig. 4—Sublimation curve for undiluted semen, Jersey bull 525009.

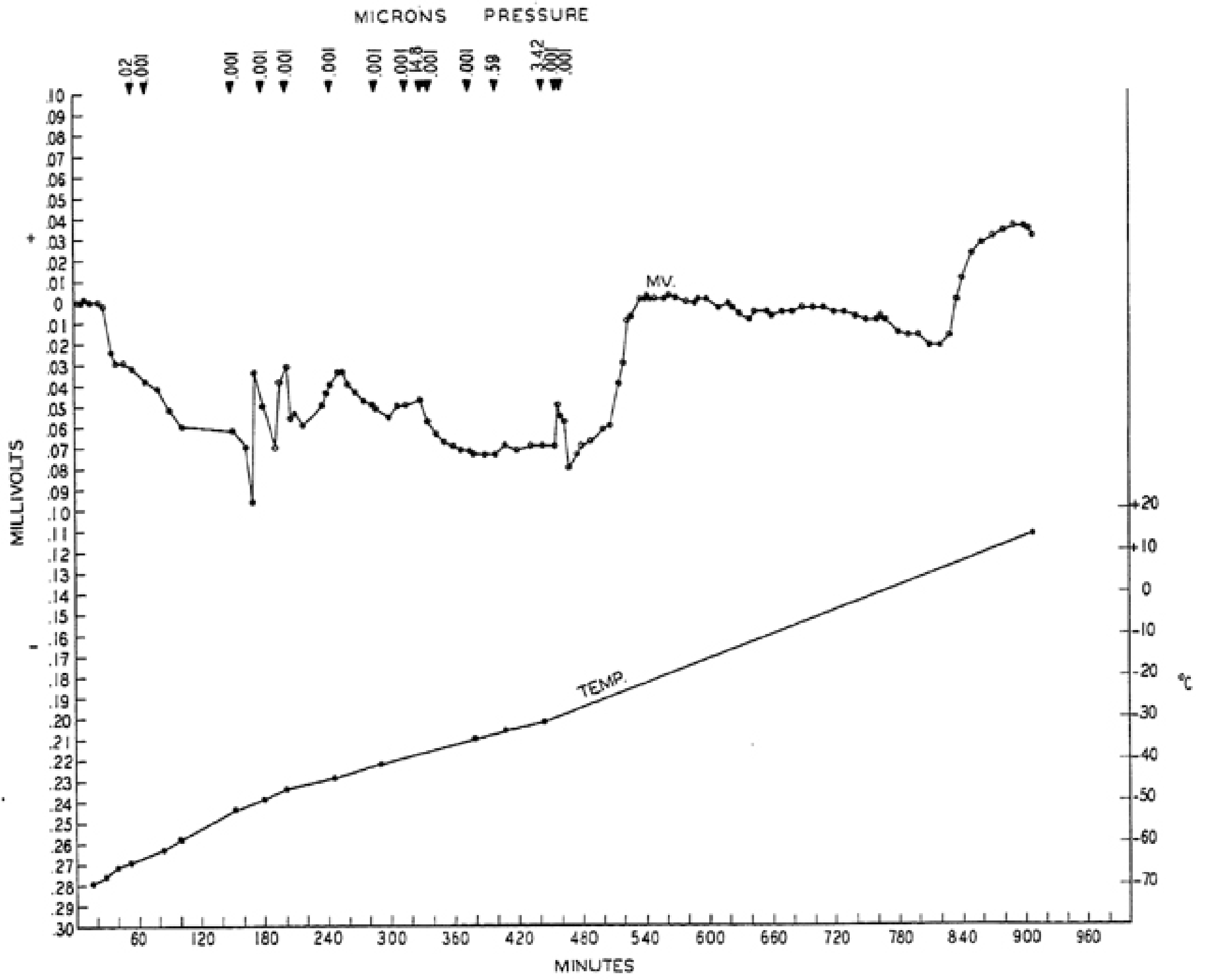

Fig. 5—Sublimation curve for undiluted semen, Holstein bull 1198129.

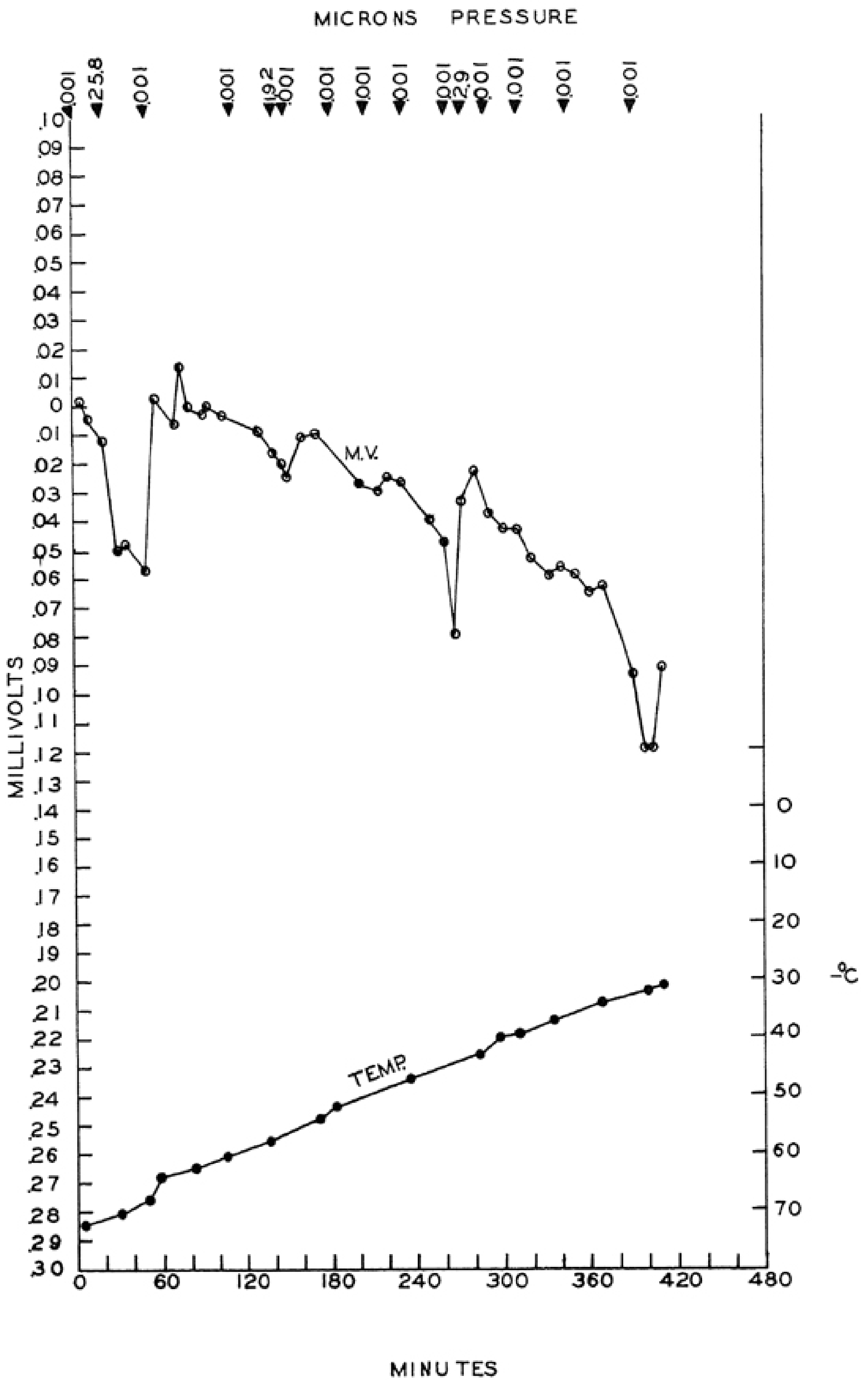

Fig. 6—Sublimation curve for undiluted semen, Holstein bull $337B_5$.

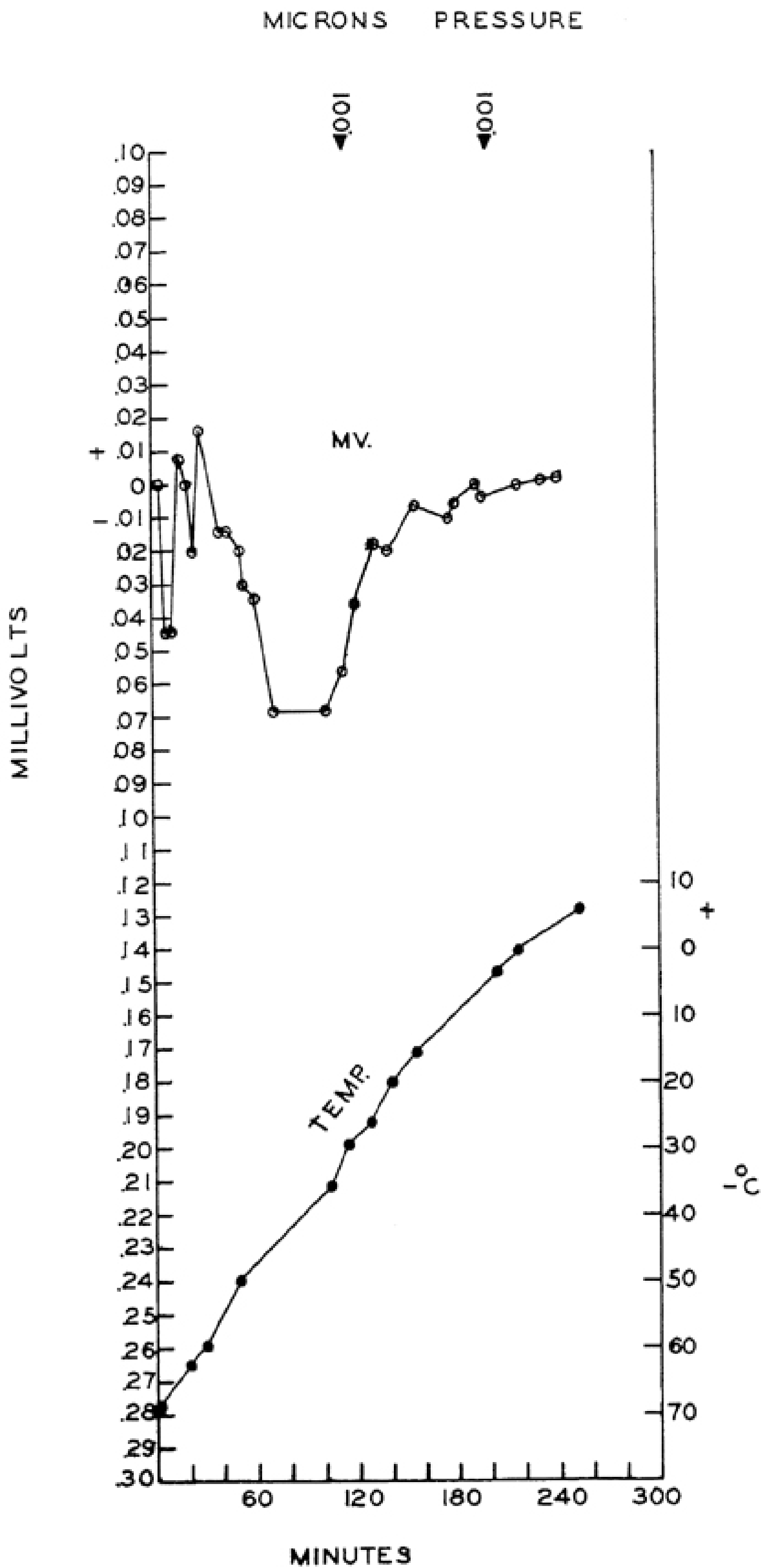

Fig. 7—Sublimation curve for potassium citrate diluted semen, Holstein bull 465B₂.

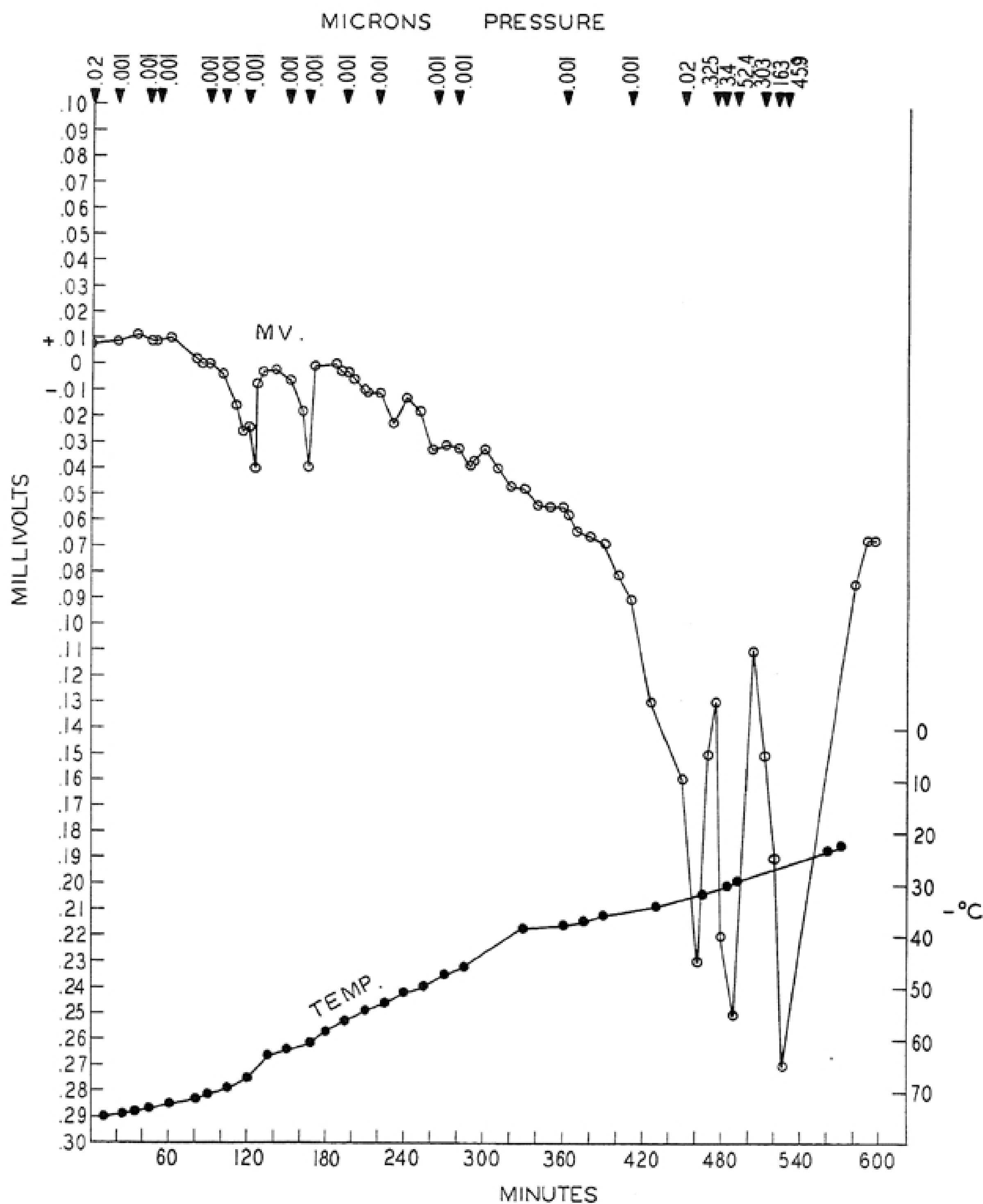

Fig. 8—Sublimation curve for potassium citrate diluted semen, Holstein bull 1283950.

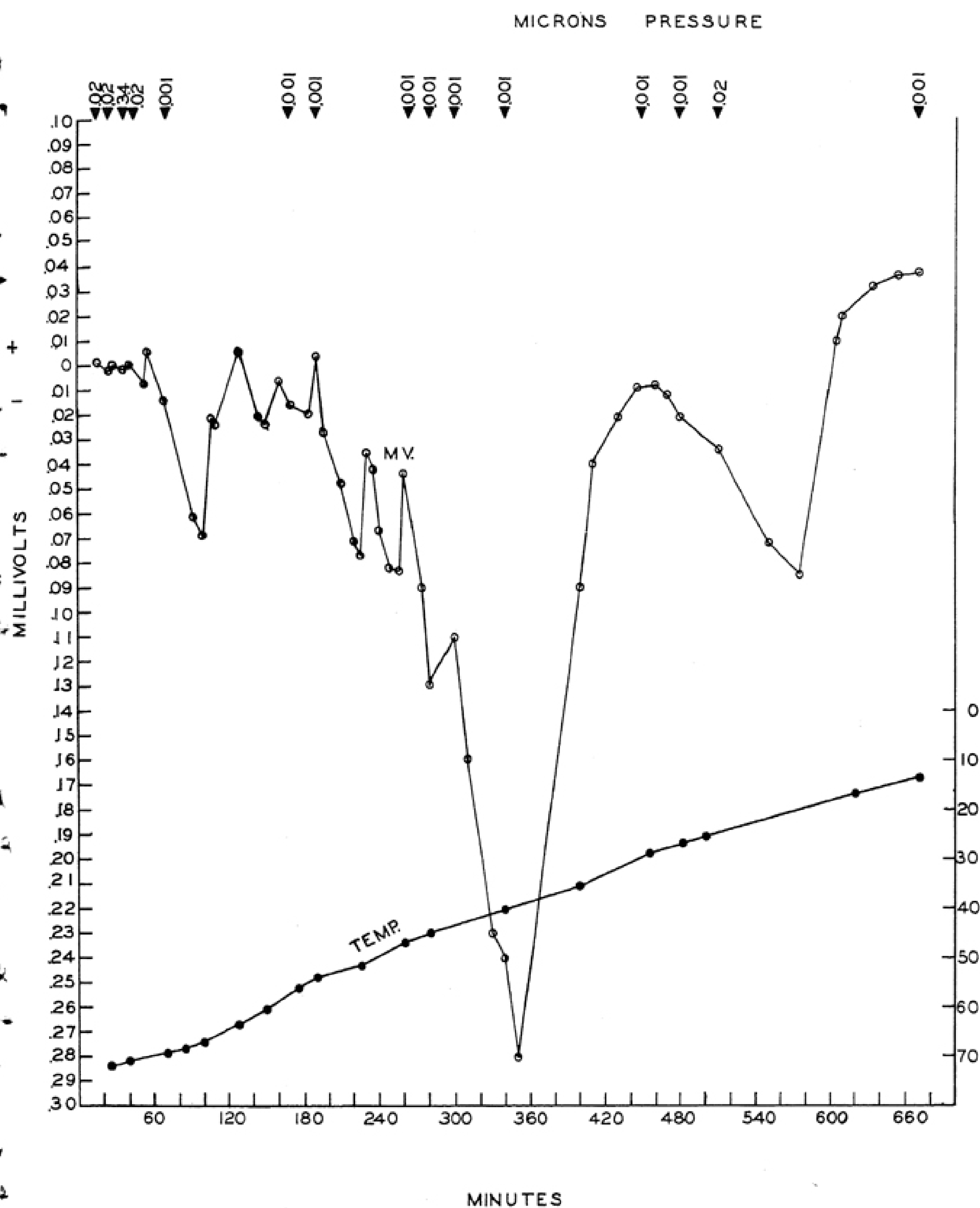

Fig. 9—Sublimation curve for potassium citrate-glycerol diluted semen, Holstein bull $337B_5$.

scope. The spermatozoa appeared normal, but no motility was seen. Other organic liquids used in this dehydration method were trimethyl amine, dimethyl amine, ethyl chloride, ethylene oxide, and methyl mercaptan, but they did not seem to dehydrate semen as readily as acetone.

Liquid-gas extraction dehydration studies included preliminary trials to determine if pressure adversely affected spermatozoa. One-half ml. of one-day-old egg yolk-sodium citrate diluted semen and 0.5 ml. of one-day-old undiluted semen were placed in separate vials inside a combustion bomb at room temperature. The pressure inside the bomb was raised to 500 psi over a 3-minute period by admitting helium. It was held at this level for 15 minutes, increased to 1000 psi over another 3-minute period, and held for 30 minutes. However, there was a slight leak and the pressure gradually decreased to 675 psi. Final release of the gas took 4 minutes. The motility of the spermatozoa in diluted semen under pressure decreased from 3+ to 3, while motility of spermatozoa in the undiluted semen was estimated to have dropped from 3 to 3-. A diluted control at room temperature was rated 3+. A dead-alive count was made using as a stain a mixture of fast green, F. C. F., and erythrocin in M/8 phosphate buffer as described by Mayer *et al.* (1951). After pressure treatment, calculations showed a 3 percent increase in the number of dead cells in undiluted semen and a 5 percent increase in dead cells in the diluted semen. Similar experiments using nitrogen and subsequent experiments using helium showed no visible effects of 1000 psi nitrogen or helium pressure on motility ratings. In one experiment, spermatozoa were kept under 700 psi helium for 100 minutes with no visible change in motility.

Carbon dioxide toxicity also was checked. Egg yolk-sodium citrate diluted semen, egg yolk-sodium citrate diluted semen containing 10 percent final concentration of glycerol, and undiluted semen were placed under 500 psi carbon dioxide, then immediately decompressed over a 15 minute period. Motility in each sample appeared depressed very slightly. Similar samples lost all motility after being exposed to 675 psi carbon dioxide for 20 minutes or to 500 psi for 30 minutes with slow decompression. However, about 90 percent of the original motility was maintained when identical samples were exposed to 500 psi of 95.2 percent nitrogen and 4.8 percent carbon dioxide for 30 minutes. Bromcresol purple indicator was added to sodium citrate diluted semen and to undiluted semen and the samples were placed under 500 psi carbon dioxide at room temperature for 30 minutes. The sodium citrate diluted semen was yellow after decompression, indicating that the pH was below 5.2 (Merck & Co., Inc., 1952). The undiluted semen was at the intermediate color stage. The sodium citrate diluted spermatozoa had lost all motility, while the undiluted semen still showed slight spermatozoan motility.

Spermatozoa, either diluted with egg yolk-sodium citrate or in undiluted semen, appeared relatively insensitive to 500 psi ethane at room temperature for periods up to 20 minutes. Upon release of the gas and examination of the cells under a microscope, no change in motility was noted. Ethane-treated cells were

stored in a refrigerator for 12 hours and when examined showed only slightly lower motility than control samples which had not been pressurized. Exposure of spermatozoa to 500 psi ethylene gas for 5 minutes stimulated motility, but when these cells were stored for 12 hours in a refrigerator, motility decreased markedly.

In a series of trials, 0.2 to 0.5 ml. egg yolk-sodium citrate diluted semen was exposed to liquid ethane (780 psi pressure, 0° C.) and liquid ethane plus helium (1700 to 1800 psi) in a 3 ml. pressure cell for 10 to 15 minutes. The pressure cell was then warmed above the critical point of ethane and the resultant gas was released. The samples still contained moisture and had decreased somewhat in motility. Two-tenths ml. of egg yolk-sodium citrate semen was placed on a small piece of cotton and exposed to ethane in the preceding manner. Upon examination the sample appeared almost dry, but no motility was observed either before or after rehydration with sodium citrate buffer. When Tween 80 (0.3 percent final concentration) was added to 0.5 ml. of semen, exposure of the sample to liquid ethane also resulted in a complete loss of spermatozoan motility.

Solid-liquid gas extraction dehydration trials were conducted by holding frozen spermatozoa in acetone at –79° C. for approximately six weeks. The acetone was repleced each 2 weeks. After the spermatozoa were thought to be dry, they were exposed to liquid ethane at -79° C. The ethane was next warmed above its critical temperature and released as a gas. The sample appeared dry, dissolved readily in water or sodium citrate buffer solution, and the spermatozoa appeared morphologically normal under the microscope. However, no motility was observed.

DISCUSSION

In comparing the freezing points of glycerol-water solutions (Figure 1) with the freezing points of glycerol-egg yolk-sodium citrate mixtures (Figure 3), it is seen that, while pure water freezes at 0° C., the egg yolk-sodium citrate diluter froze at about –¼° C. Other differences in the two freezing curves also are readily apparent. At 60 percent glycerol, the glycerol-water freezing point diagram reached a minimum of –46.5° C. In the case of egg yolk-sodium citrate mixtures, as increasing concentrations were added, freezing occurred at temperatures increasingly below those of the corresponding glycerol water solutions. The lowest freezing point detected was –55° C. with a 50 percent glycerol, 50 percent egg yolk-sodium citrate mixture. In contrast, the glycerol-water solution containing 50 percent glycerol freezes at –29.5° C. (Figure 1). The viscosity of the egg yolk sodium citrate-glycerol mixtures seemed to increase gradually upon cooling until about –60° C. was reached, at which temperature a stirring rod would become firmly fixed in the sample. Since no freezing was noted with con-

centrations of glycerol greater than 50 percent, the possibility is suggested that the egg yolk-sodium citrate-glycerol mixture might change into the vitreous state in the region of 60° C.

Helium or nitrogen atmospheres of up to 100 psi pressure had little, if any, effect on spermatozoa. The apparent toxicity of carbon dioxide was probably due to the resultant decrease in pH since 500 psi carbon dioxide for 30 minutes at room temperature lowered the pH in a sodium citrate diluted semen sample to less than 5.2. Brief exposure to ethylene gas at 500 psi pressure stimulated spermatozoan motility, but upon storage, the motility decreased markedly. Ethane gas appeared relatively non-toxic.

Liquid ethane was found to have little dehydrating ability. Being a saturated hydrocarbon, its water miscibility would be expected to be low. The ethane was usually found to be non-toxic to spermatozoa in normal media, but when Tween 80 (surface tension depressant) was added in a non-lethal quantity, pressure treatment with liquid ethane resulted in a complete loss of spermatozoan motility. These results indicate that the normal non-toxicity shown by liquid ethane is due to its immiscibility with aqueous media.

In solid-liquid extraction trials, acetone dehydrated spermatozoa, but upon rehydration, loss of spermatozoan motility and denaturation of the diluter was noted. The spermatozoa may have been killed by the acetone at -79° C., or they may have been killed because of inadequate methods of acetone removal.

Solid-liquid-gas extraction appeared to dehydrate spermatozoa. Upon rehydration, the samples dissolved readily with no visible signs of denaturation, but no motile spermatozoa were seen. Future work with various liquids and gases might be profitable either in attempting to dry spermatozoa or in drying other materials.

In experiments similar to those reported by Yushchenko (1957), spermatozoa were extremely difficult to centrifuge into mixtures of freons and heptane (practical). Yushchenko reported that he used specific gravities of 1.18 to 1.44. However, in experiments performed by the authors, spermatozoa could not be forced down by centrifugation for up to 30 minutes at 15,500 rpm (15,500 rpm = 31,000 x gravity) until the freons-heptane mixture was reduced in specific gravity to about 1.15. The addition of Tween 80 did not allow spermatozoa to be centrifuged into a 1.25 specific gravity mixture. The motility of spermatozoa in the mixtures of freons and heptane (practical) was not greatly reduced. In fact, the spermatozoa could be allowed to remain in a clump in the organic mixture for at least 15 minutes at room temperature with no drastic reduction in motility. This apparent non-toxicity may be due to the low miscibility of the organic mixture with water. In only a small proportion of the trials was there any survival after freezing the spermatozoa. It must be pointed out that pure n-heptane was not used. However, the heptane (practical) contained 50 percent n-heptane and seemed to be reasonably non-toxic to spermatozoa under the conditions used.

A reason for failure of the freeze-drying method in preserving spermatozoa is sometimes postulated to be the failure to remove glycerol and the toxicity of glycerol in the high concentration which remains. If this is true, before freeze-drying of spermatozoa can be successful, the spermatozoa must either be successfully frozen without glycerol or the glycerol present will have to be decreased in quantity or removed, or its toxicity prevented (possibly by converting it into a non-toxic product). Attempts were made to reduce the amount of glycerol present by centrifugation of the spermatozoa before freezing, by centrifugation of the spermatozoa into a mixture of freons and heptane (practical), by absorbing the glycerol into a wood splint during lyophilization, and by trying to aid its removal by using a potassium permanganate trap during dehydration. Freeze-drying in all cases was unsuccessful in that no motile spermatozoa were seen after the sample was taken to apparent dryness. Dehydration of spermatozoa after replacement of glycerol with methyl alcohol or after partial replacement with propylene glycol was also successful. However, in these cases lyophilization could only be performed at temperatures warmer than the eutectic point of the sample.

To dry below the eutectic point, lyophilization should be performed at a temperature colder than -55° C. The exact temperature is not known, but since spermatozoa will store at -79° C, this temperature is probably sufficiently cold. Freeze-drying with the Stokes 102-L cryochem apparatus for two weeks at pressures averaging about 200 microns Hg. and reaching as low as 75 microns Hg. did not dry spermatozoa. It is not known why the spermatozoa declined in motility during this period although undetected sample temperature fluctuations are a possibility. Since the vapor pressure of pure ice is only 0.56 micron Hg. at -79° C. (Hodgman, 1949) and the salts present in semen lower the vapor pressure below this, complete drying was not expected. Albright, *et al.* (1958) do not state the amount of vacuum developed by their freeze-drying system. However, since they froze semen in a dry ice bath and began lyophilization as the temperature became colder than -50° C, it is doubtful that they removed much water in the 45 minute lyophilization period given.

Figures 4 to 9 show sublimation curves developed with the aid of a measuring thermocouple located inside the sample and a reference thermocouple in the constantly warming fluid bath. When a substance sublimed, the measuring thermocouple became colder than the reference thermocouple (as much as 7° C in Figure 9) and a voltage was produced. As plotted, negative millivolts indicate the sample was colder than the bath temperature, i.e., a substance was subliming. After a phase completely sublimed, and provided a new phase had not yet started subliming, the temperature inside the sample tube warmed to bath temperature.

Figure 4, 5, and 6 are sublimation curves for raw semen samples from three different bulls. The bath temperatures at which the voltage curves reached mini-

ma can be used in comparing the samples. In Figure 4 many minima are shown, indicating that many phases sublimed as the sample warmed. The three most distinct minima occurred at bath temperatures of -67° C, -36° C, and -28½° C to -27½° C. In Figure 5, the various phases are not so distinct. There appears to be a plateau in the curve for 8 minutes at a bath temperature of -65½° C to -65° C and further sublimation in the general ranges of -50½° C to -46° C and 36½° C to -27½° C, with a final phase at +4° C to +5½° C. This is the only sample in which the electromotive force was recorded at bath temperatures above 0° C. The sample described by Figure 6 was not taken to dryness; however, it appears that fractions vaporized at bath temperatures of -68° C, -44° C, and -32° C to -31½° C.

Figures 7 and 8 describe lyophilization of semen which was diluted with equal parts of M/10 potassium citrate before freezing. The sample represented by Figure 7, which was warmed more rapidly than the other, shows rather distinct minima at the following bath temperatures: -67° C to -65½° C, -61½° C, -44° C to -36° C. Figure 8 shows vaporization peaks at -67° C, -61° C, and in the range of -32° C to -26½° C. There are three minima between -32° C and -26½° C, but vacuum leaks occurred in the system at this range and all three of these depressions probably represent sublimation of one fraction.

Figure 9 represents the freeze-drying of semen diluted with potassium citrate plus glycerol. Several minima in the voltage curve are shown, including three distinct ones at bath temperatures of -67½° C, -39½° C, and -20° C. The minimum at -20° C is interesting in that the temperature inside the sample tube had risen to within ¼° C of the bath temperature. Then another fraction began vaporizing and the measuring junction inside the sample tube cooled to more than 2° C below the bath temperature. Preliminary trials indicate that the point at which this phase came off (approximately 10 to 15 percent residual moisture) is the approximate dehydration death point of bovine spermatozoa as shown by motility observations on rehydrated samples.

Future fractional sublimation work of this type could lead to a better understanding of why death occurs during freeze-drying of spermatozoa. It seems reasonable to expect, and Figures 4, 5, and 6 indicate, that a considerable variation in the sublimation curves of raw and diluted semen from different bulls and /or ejaculates will be found. Possibly certain types of curves will be correlated with ability of the spermatozoa to withstand freezing or even with fertility.

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